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Title:
Identifying genes controlling Candida albicans resistance to neutrophils

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Candida albicans resistance to neutrophils

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ABSTRACT

Each year, *Candida albicans* causes ~700,000 cases of invasive life-threatening candidemia with a mortality rate of 40%. The opportunistic pathogen *C. albicans* is the fourth most common cause of nosocomial infections. While neutrophils are the first line of defence against invasive *C. albicans*, the yeast has developed several strategies to escape neutrophil killing effectively. The project aims to identify *C. albicans* genes that are important for the survival of neutrophil antifungal responses. These could then be exploited as future drug targets to stop *C. albicans* immune evasion.

A high-throughput fluorescence-based killing assay was established and used to screen 384 conditional mutants from the GRACE^{TM} library, the largest publicly available *C. albicans* mutant library. Neutrophil-like cells, PLB-985, were used in the initial screen. Any mutants that were susceptible to PLB-985 killing were further validated with primary human neutrophils. Validated mutants were analysed in a series of functional assays to identify potential pathways and mechanisms involved in immune evasion.

25 out of 384 *C. albicans* mutants were found to be susceptible to PLB-985 killing. After four further validations with neutrophils, 5 mutants were found to be vulnerable to neutrophil killing. These mutants comprise *brr2, taf7, noc3, tfb3* and *smt3* with approximately 38%, 44%, 27%, 28% and 29% reduction in cell viability relative to the *C. albicans* WT control, respectively. Functional assays further revealed that the five identified genes might play essential roles in *C. albicans* immune evasion. For example, the *noc3* mutant appeared to be a weak inducer, and the *tfb3* mutant appeared to be a strong inducer of reactive oxygen species and neutrophil extracellular traps production. Therefore, these genes may represent good therapeutic targets for candidemia.

DEDICATION AND ACKNOWLEDGEMENTS

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AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ALS1	Agglutinin-like protein 1 precursor
BSA	Bovine Serum Albumin
C. albicans	*Candida albicans*
CARD9	Caspase recruitment domain-containing protein 9
CD	Cluster of differentiation
Cek1	Extracellular signal-regulated kinase 1
CFW	Calcofluor White
CGD	Chronic granulomatous disease
CLRs	C-type lectin receptors
CPH1	Phytochrome-like protein Cph1
CR	Complement receptor
Cu^{2+}	Copper ions
ddH_2O	Double distilled water
DMF	N, N-Dimethylformamide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERG11	Lanosterol 14-alpha demethylase
ERG13	Hydroxymethyglutaryl-CoA synthase
ERK	Extracellular signal-regulated kinase
FBS/FCS	Foetal bovine serum/foetal calf serum

FcγRs	Fc-gamma receptors
FDA	U.S. Food and Drug Administration
FHL-5	Familial hemophagocytic lymphohistiocytosis type 5
FKS1	1,3-beta-glucan synthetase component FKS1
G-CSF	Granulocyte colony-stimulating factor
GIT	Gastrointestinal tract
GRACE™	Gene replacement and conditional expression
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
hCAP-18	Human cathelicidin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGC1	Hypha-specific G1 cyclin-related protein 1
HIV	Human immunodeficiency virus
HOCL	Hypochlorous acid
Hog1	Mitogen-activated protein kinase HOG1
HRP	Horseradish peroxidase
HSA	Human Serum Albumin
HSCs	Haematopoietic stem cells
HWP1	Hyphal wall protein 1
ICAM	Intracellular adhesion molecule
ICL1	Isocitrate lyase 1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IRAK	Interleukin receptor associated kinase

KA	Killing assay
LAD	Leukocyte adhesion deficiency
LFA	Lymphocyte function-associated antigen
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance mutation 1
MEK	Mitogen-activated protein kinase kinase
MES-NaOH	M 2-(*N*-morpholine)-ethane sulfonic acid-sodium hydroxide
MI	Morphology Index
MIP	Macrophage inflammatory protein
MLS1	Malate synthase 1
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response 88
n	Number of biological replicates
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NOC3	Nucleolar complex-associated 3
NOX2	NADPH oxidase
NPI	NETs per image
$O_2^{\cdot-}$	Superoxide anions
OD	Optical Density

P/S	Penicillin-Streptomycin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PKC	Protein Kinase C
PLB-985	Promyeloblast leukaemia HL-60
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear leukocytes
PR3	Proteinase 3
PRRs	Pattern recognition receptors
PSGL-1	P-selectin glycoprotein ligand-1
PTX3	Pentraxin 3
Q	L-Glutamine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Saps	Secreted aspartyl proteinases
SD	Standard deviation
SEM	Standard error of mean
SOD	Superoxide dismutase
Syk	Spleen tyrosine kinase
TAF7	Transcription initiation factor TFIID subunit 7
TFB3	Transcription factor B subunit 3
Th	T-helper cells
TLRs	Toll-like receptors

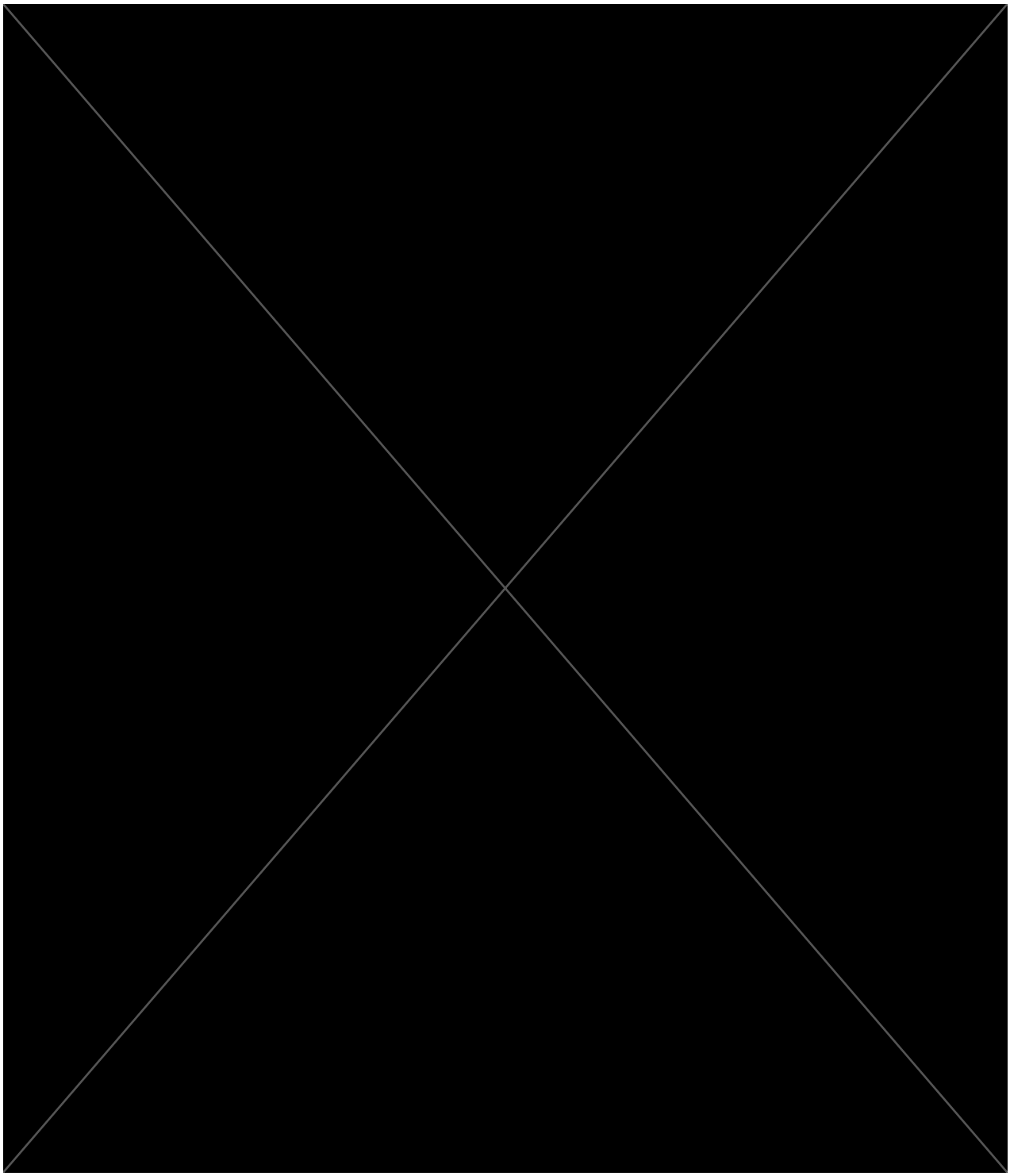
TNF	Tumour necrosis factor
TRAF	Tumour necrosis receptor-associated factor
TUP1	Transcriptional repressor TUP1
WBC	White blood cell
WT	Wild type
YE	Yeast Extract
Zn^{2+}	Zinc ions

CHAPTER 1: INTRODUCTION

1.1 *CANDIDA ALBICANS* IS A MAJOR FUNGAL PATHOGEN OF HUMANS

1.1.1 *CANDIDA ALBICANS* IN THE HEALTHY HOST

Candida albicans is one of humans' most common opportunistic fungi, affecting millions worldwide (Bongomin et al., 2017). It is a member of the human microbiota and a commensal of the skin, oral cavity, and gastrointestinal and genitourinary tracts in healthy individuals (Brown et al., 2012, Erdogan and Rao, 2015, Pappas et al., 2018). Like other members of the human microbiota, the *C. albicans* population is tightly regulated and kept under control by our immune system (Figure 1.1), particularly by the innate immune cells such as macrophages and neutrophils (Abbas et al., 2018, Gazendam et al., 2016, Qin et al., 2016, Wibawa, 2012). A weak immune system can lead to the overgrowth of *C. albicans* and provide this microbe with an opportunity to turn into a pathogenic fungus by allowing it to express its virulence factors to establish an infection. Therefore, immunocompromised individuals are particularly vulnerable to *C. albicans* infections, also known as candidiasis (Brown et al., 2012, Erdogan and Rao, 2015, Gazendam et al., 2016, Martins et al., 2014).



1.1.2 *C. ALBICANS* IS THE 4TH MOST COMMON CAUSE OF NOSOCOMIAL BLOODSTREAM INFECTIONS

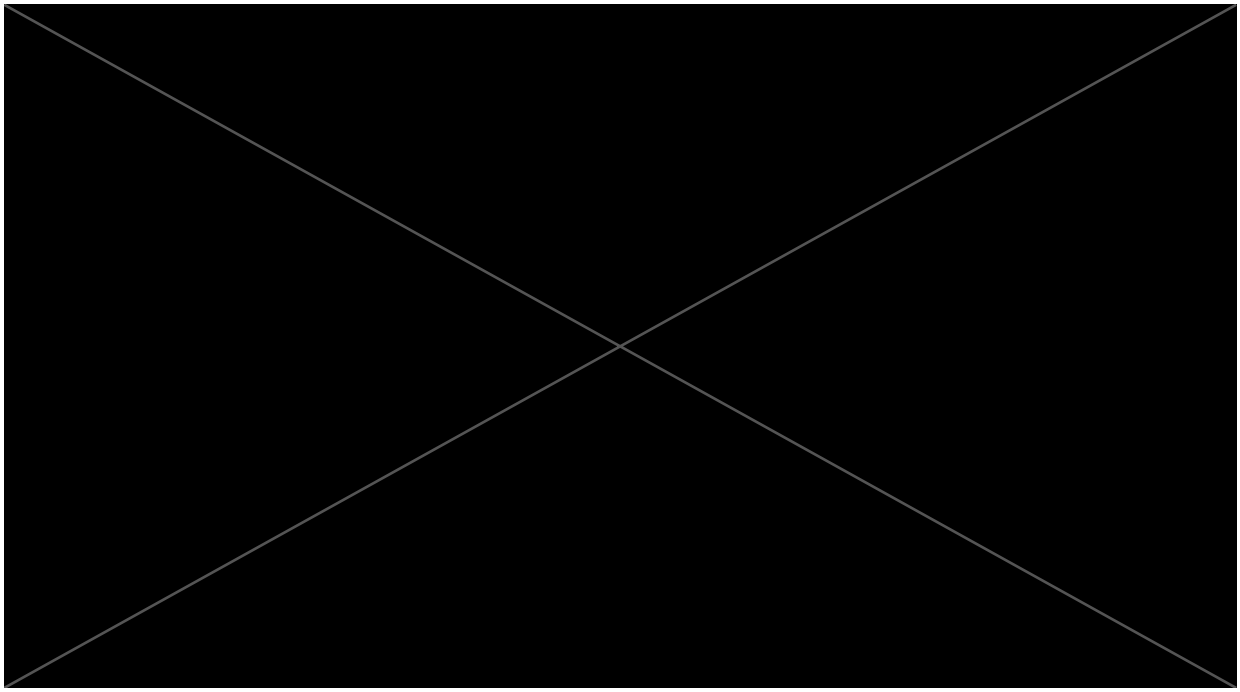
Candidiasis is a broad-spectrum disease (Figure 1.2) commonly associated with *C. albicans*. It can affect different parts of our body, from superficial sites to deep systemic (invasive) infections (Martins et al., 2014, Pappas et al., 2018, Vazquez-Gonzalez et al., 2013).

1.1.2.1 SUPERFICIAL CANDIDIASIS

Superficial candidiasis is a localised infection that is limited to the skin surface (cutaneous candidiasis), mucous membrane (mucosal candidiasis) and genital area (Figure 1.2) (Martins et al., 2014, Vazquez-Gonzalez et al., 2013). Superficial candidiasis is a reasonably common infection. For example, 5-7% of babies were estimated to develop oral candidiasis, while around 75% of women were estimated to have at least one episode of vulvovaginal candidiasis in their lifetimes (Patil et al., 2015, Goncalves et al., 2016).

Superficial candidiasis risk factors:

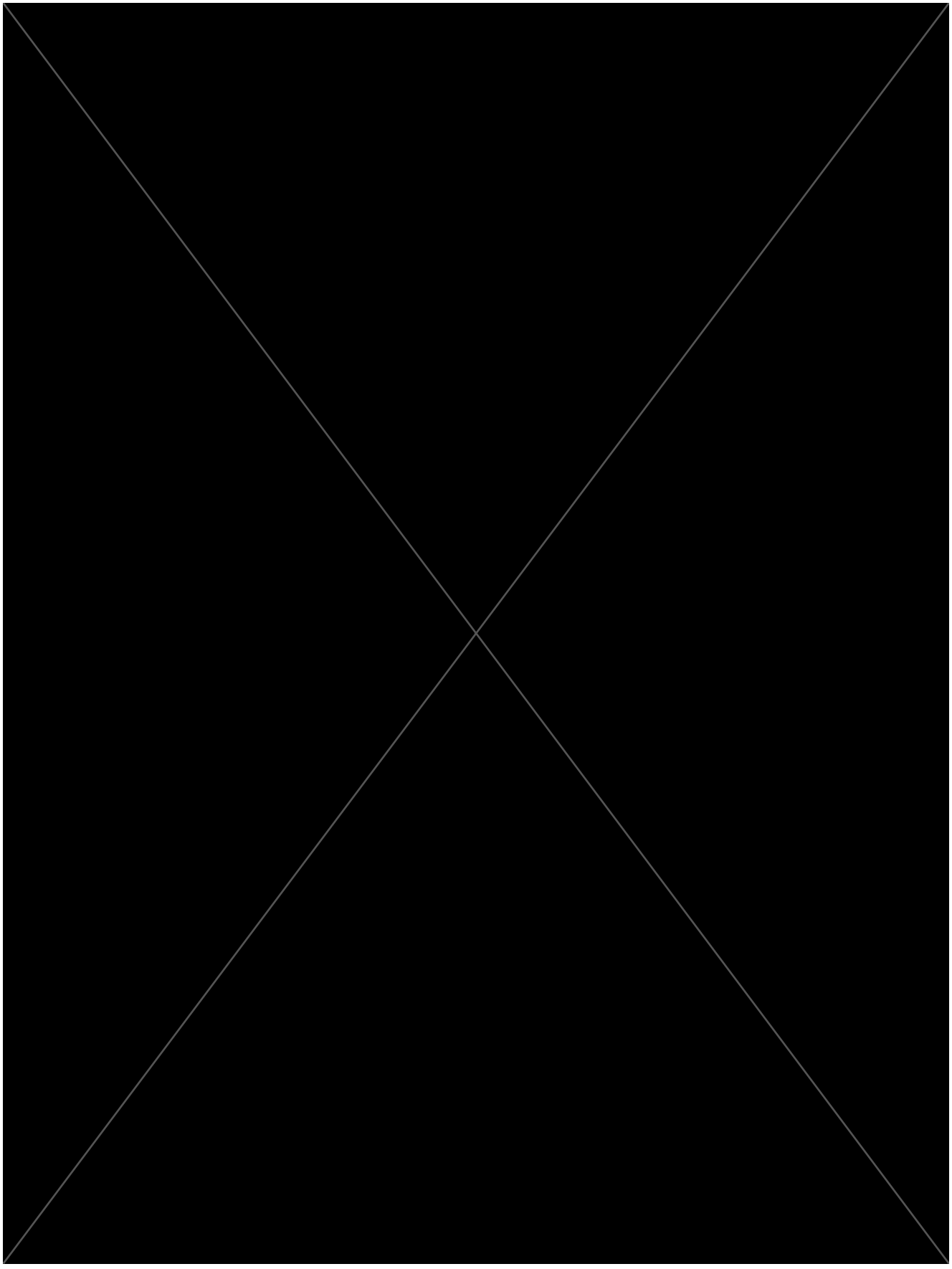
One of the significant underlying risks of acquiring superficial candidiasis is having a compromised immune system. For example, people who are living with human immunodeficiency virus (HIV) or acquired immune deficiency syndrome (AIDS) often suffer from oesophageal candidiasis (Anwar et al., 2012, de Repentigny et al., 2004). Similarly, oropharyngeal candidiasis is often seen and commonly associated with morbidity and mortality in cancer patients on immunotoxic therapies, such as cytotoxic chemotherapy and radiotherapy (Jayachandran et al., 2016, Maksymiuk et al., 1984). Interestingly, the use of oral contraceptives has been shown to associate with the risk of developing vulvovaginal candidiasis (Spinillo et al., 1995).



1.1.2.2 INVASIVE CANDIDIASIS

Invasive candidiasis is the most severe form of *C. albicans* infection (Bongomin et al., 2017, Brown et al., 2012). There were estimated to be ~700,000 cases worldwide in 2017, where 5,142 cases were reported in the UK in 2016 (Bongomin et al., 2017, Pegorie et al., 2016). Unlike superficial candidiasis that locally affects the mouth and throat or vaginal tract, invasive candidiasis can evade and spread to other parts of the body, such as the bloodstream, brain, heart and lungs (Brown et al., 2012, Kullberg and Arendrup, 2015).

Candidemia, defined as the presence of *Candida* species in the bloodstream (Figure 1.2b and Figure 1.3), is the most common form of invasive candidiasis, with a more than 40% death rate (Bongomin et al., 2017, Kullberg and Arendrup, 2015). *C. albicans* is one of the fourth most common causes of nosocomial bloodstream infection, commonly observed in immunocompromised individuals, including critically ill patients, older people and premature neonates (Bassetti et al., 2006, Bongomin et al., 2017, Wisplinghoff et al., 2004).



Candidemia risk factors:

Not having efficacious antifungal therapeutics, the incidence of nosocomial candidemia continues to rise over the past decades (Bongomin et al., 2017, Fridkin and Jarvis, 1996, Wisplinghoff et al., 2004). The increasing incidence of candidemia is thought to be due to the increase of risk factors (Figure 1.4) (Martins et al., 2014).

Like superficial candidiasis, a compromised immune system is one of the main risk factors for acquiring candidemia (Kullberg and Arendrup, 2015, Martins et al., 2014, Yapar, 2014). For example, people who suffer from immunodeficiency conditions, such as CARD9 deficiency (mutations in a gene called caspase recruitment domain family member 9) and chronic granulomatous disease (CGD, an inherited disorder that results in phagocyte dysfunction) have a greater risk of having candidemia (Alves de Medeiros et al., 2016, Drewniak et al., 2013). Similarly, people who are taking immunosuppressive or immunotoxic therapies, such as those who suffer from autoimmune diseases, received transplantations and cancer patients, are also vulnerable to candidemia (Golecka et al., 2006, Li et al., 2017, Maksymiuk et al., 1984, Vaquero-Herrero et al., 2020, Yapar, 2014).

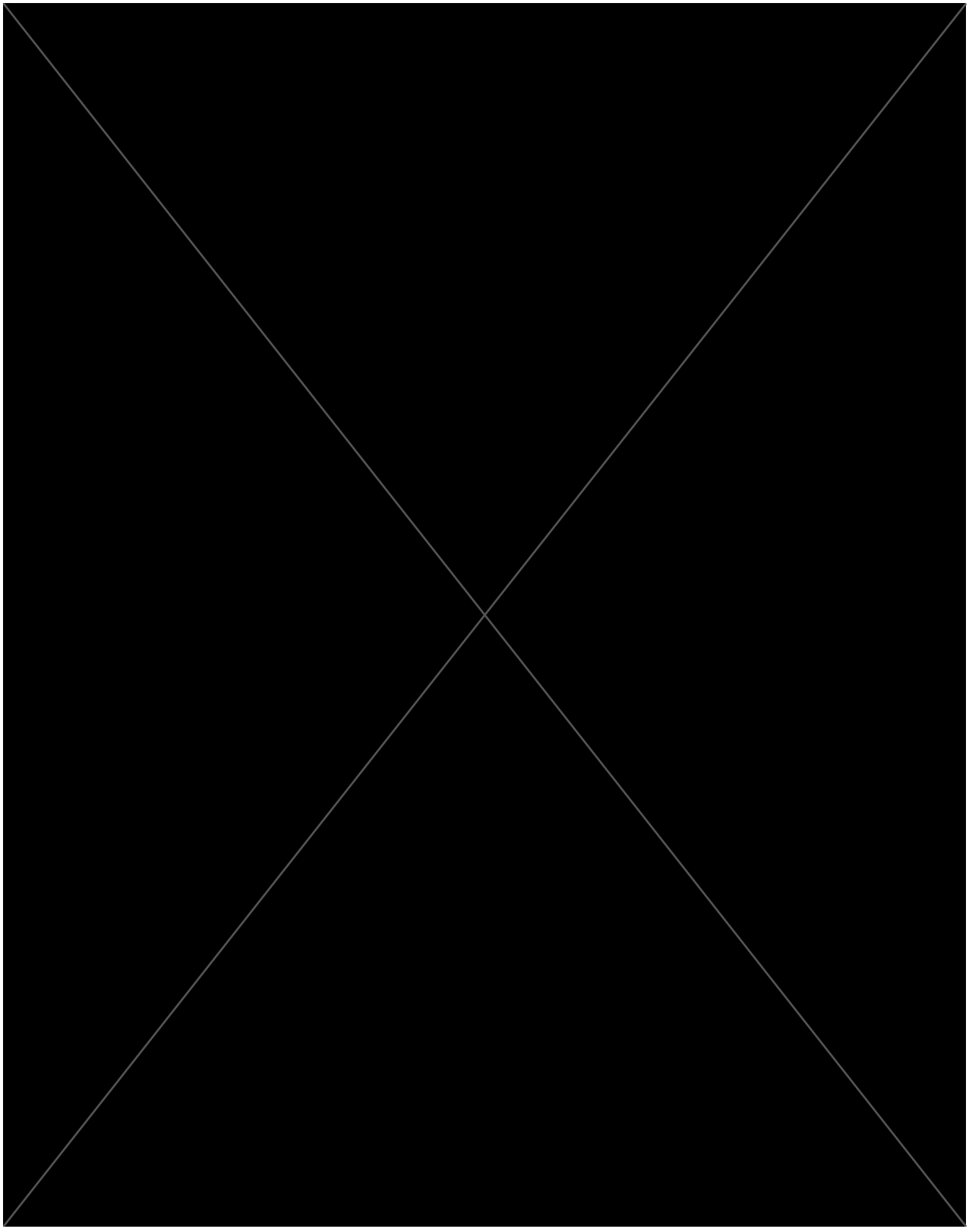
The use of broad-spectrum antibiotics is another important risk factor of candidemia due to their ability to disturb the homeostasis of the human microbiota and promote *C. albicans* colonisation (Seelig, 1966, Shankar et al., 2015). A healthy host-microbiome helps to keep *C. albicans* numbers in control, in addition to a healthy immune system (Stefanaki, 2019). For example, studies showed that lactobacilli (Gram-positive bacteria), common members of the human microbiome, could inhibit the growth of *C. albicans* and its virulence factors, such as adherence ability, hyphae transformation and biofilm formation (Jang et al., 2019, Matsuda et al., 2018).

Invasive medical interventions (i.e., procedures that purposely invade or access the body, usually via incision or puncture), such as implanting cardiac devices or inserting catheters, can provide easier access for *C. albicans* to enter the bloodstream. At the same time, these devices also provide surfaces for *C. albicans* to form biofilms on (Kojic and Darouiche, 2004, Yapar et al., 2011).

Diabetes mellitus, a chronic metabolic disorder, is another high-risk factor for obtaining candidemia (Bader et al., 2004, Calvet and Yoshikawa, 2001, Ostrosky-Zeichner et al., 2002). People with diabetes mellitus suffer from chronic hyperglycaemia, vascular remodelling and a dysfunctional immune system (such as impaired neutrophil functions) (Calvet and Yoshikawa, 2001, Delamaire et al., 1997, Rodrigues et al., 2019). These are favourable conditions for *C. albicans* to thrive in. For example, high blood sugar levels provide a rich source of nutrients and energy for *C. albicans* metabolism, whereas vascular remodelling increases accessibility to the bloodstream (Bader et al., 2004, Rodrigues et al., 2019). Also, diabetic patients require regular blood glucose level checks and daily insulin injections, which are risk factors (Bader et al., 2004, Calvet and Yoshikawa, 2001, Delamaire et al., 1997, Rodrigues et al., 2019).

Candidemia does not usually spread directly from person to person. However, patients with high-risk factors can acquire candidemia due to poor hygiene in healthcare settings (Pappas et al., 2016, Pfaller and Diekema, 2007). For example, many studies showed frequent *Candida* carriage on the hands of healthcare professionals, which may be a predisposing factor for nosocomial candidemia (Lupetti et al., 2002, Strausbaugh et al., 1994, Yildirim et al., 2007). Therefore, it is crucial to ensure a high standard of cleanliness and hygiene when interacting with patients or performing invasive procedures. To further prevent candidemia, high-risk

patients may benefit from antifungal prophylaxis (Pappas et al., 2016, Pfaller and Diekema, 2007).



Candidemia clinical presentations:

People who develop candidemia do not ordinarily present infection-specific symptoms and often already suffer from other medical conditions. This means their existing conditions' symptoms can overshadow candidemia (Antinori et al., 2016, Pappas et al., 2018). However, fever and chills that do not improve after antibiotic treatment are the most common presentations of candidemia (Antinori et al., 2016). Candidemia can also cause septic shock, and thus patients may present with symptoms such as hypotension, tachycardia, and tachypnoea (Guzman et al., 2011, Kollef et al., 2012). Other symptoms can present if candidiasis spread to other parts of the body, such as the heart, bones and brain (Antinori et al., 2016, Kullberg and Arendrup, 2015, Pappas et al., 2018). Therefore, it can be challenging to diagnose candidemia based on symptomatic presentation.

Diagnosis of candidemia:

The diagnostic gold standard for candidemia is blood cultures, which detect the presence of *Candida* in the bloodstream (Kullberg and Arendrup, 2015, Pappas et al., 2016). However, its overall sensitivity is only ~50%, with the limit of ≤1 colony-forming unit/mL and turnaround times ranging from 1 to ≥7 days (Berenguer et al., 1993, Clancy and Nguyen, 2013, Clancy and Nguyen, 2018, Pappas et al., 2016, Pfeiffer et al., 2011). However, many innovative culture-independent diagnostic tests are now entering into clinical practice as adjunctive tools. This includes *Candida*-antigen and -antibody detection (such as mannan, β-D-glucan and anti-mannan IgG) with ~83% sensitivity and ~86% specificity, Polymerase Chain Reaction (PCR) test (T2Candida Panel) with at least 85% sensitivity and 98% specificity, and Mass Spectrometry (MALDI-TOF) with at least 90% sensitivity and specificity (Bader et al., 2011, Bille et al., 2012, Iriart et al., 2012, Kullberg and Arendrup, 2015, Mikulsa et al., 2010, Mylonakis et al., 2015, Wei et al., 2020).

Treatment of candidemia:

Candidemia patients require a specific type and dose of antifungal treatments, which usually depend on the individual patient factors, such as age, immune status and other relevant comorbidities, including neutropenia and renal failure (Pappas et al., 2016). **Echinocandins** are often used as initial therapy for adult patients (Pappas et al., 2016). They inhibit β-(1,3)-D-glucan synthase function, resulting in the weakening of the fungal cell wall, which can lead to osmotic lysis (Odds et al., 2003, Pappas et al., 2018). Antifungal azoles, such as **Fluconazole**, are often used as first-line agents for step down therapy (Pappas et al., 2016). They target human cytochrome P450-Erg11p, an enzyme that catalyses the removal of the 14α-methyl group of lanosterol. These agents inhibit 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway. This causes the disturbance of fungal membrane permeability and synthesis (Odds et al., 2003, Pappas et al., 2018, Vanden Bossche et al., 1995). Polyene antifungal agents, such as **Amphotericin B,** are other first-line agents for step down treatment (Pappas et al., 2016). They directly bind to ergosterol and cause polar pore formation in the fungal membranes leading to osmotic lysis due to loss of ions, such as potassium and hydrogen ions (Ellis, 2002, Odds et al., 2003, Pappas et al., 2018).

Antifungal resistance remains one of the biggest challenges when treating candidemia. Over time, *C. albicans* has developed effective strategies to avoid being killed by these antifungal treatments. For example, echinocandin-resistant *C. albicans* was found to have *FKS1* mutations, a gene associated with cell wall biogenesis (Balashov et al., 2006, Ben-Ami et al., 2011, Desnos-Ollivier et al., 2008). Likewise, fluconazole-resistant *C. albicans* was found to overexpress *ERG11*, a gene associated with ergosterol production, and to overexpress *MDR1*, a gene that encodes drug efflux pumps (Dunkel et al., 2008, Flowers et al., 2012, Whaley et al., 2017). Therefore, there is an urgent need to develop novel antifungal therapeutics.

1.1.3 *C. ALBICANS* EXPRESSES A SUITE OF VIRULENCE FACTORS TO ESTABLISH AN INFECTION

1.1.3.1 ADHERENCE

The ability to adhere to the host cell surface is essential for *C. albicans* to colonise and establish infection successfully. Studies have shown a clear link between adherence ability and virulence of *C. albicans* (McCullough et al., 1996). For example, *C. albicans* strains that have increased ability to adhere to epithelial cells *in vitro* were demonstrated to have increased ability to cause infection in animal models (Ghannoum and Elteen, 1986, Rotrosen et al., 1986). It has been shown that *C. albicans* in the hyphal stage has a greater ability to adhere to host tissues than the yeast phase (Fu et al., 2002, Tsuchimori et al., 2000). *C. albicans* surface molecules called adhesins, mediate *C. albicans* binding to the host surface or its neighbouring cells (Cannon and Chaffin, 1999, Wibawa, 2016). Several genes have been proposed to be involved in adhesin production, such as *ALS1* (encoding for Als1p, important for epithelial and endothelial cell adhesion) and *HWP1* (encoding Hwp1p, important for epithelial cells) (Fu et al., 2002, Fu et al., 1998, Kumamoto and Vinces, 2005, Tsuchimori et al., 2000).

1.1.3.2 DIMORPHISM

C. albicans can exist in the unicellular yeast phase or multicellular and filamentous hyphal phase (Pappas et al., 2018, Wibawa, 2016). The transition (or dimorphism) between these two phases depends on several environmental conditions, such as temperature, pH and chemical constituents (Han et al., 2011, Kumamoto and Vinces, 2005, Molero et al., 1998, Wibawa, 2016). Several genes are important in the morphogenesis of *C. albicans,* such as *CPH1, EGF1, HGC1* and *TUP1*. Deletion of MAPK pathway members (*CPH1* and *EGF1*) and the G1 cyclin related protein Hgc1 have been shown to halt filamentous growth, while deletion of *TUP1* has

been shown to induce hyphal growth (Braun and Johnson, 1997, Braun and Johnson, 2000, Han et al., 2011, Lo et al., 1997, Zheng et al., 2004). The hyphal phase has also been suggested to be more virulent than the yeast phase (Lo et al., 1997, Tsuchimori et al., 2000). For example, a *C. albicans egf1Δ/Δ cph1Δ/Δ* mutant showed a significant reduction in pathogenicity in mice (Lo et al., 1997). Nevertheless, both morphologies are important in superficial and invasive *C. albicans* infections. Due to its ability to penetrate a deeper tissue layer, the hyphal form is considered to be important in tissue invasion, such as from the GI mucosal layer into vascular endothelium and reaching the bloodstream (Kullberg and Arendrup, 2015, Pappas et al., 2018, Wilson et al., 2016). The levels of adhesins and hydrolytic enzymes are also upregulated in the hyphal phase (Kumamoto and Vinces, 2005). Meanwhile, the yeast phase has been reported to play an important role in candidemia and *C. albicans* dissemination due to its budding ability and small size, for example, budding from hyphae to enter blood circulation, and ultimately spreading to various organs: bone, lungs, heart, liver and kidneys (Kullberg and Arendrup, 2015, Pappas et al., 2018, Wibawa, 2016, Wilson et al., 2016).

1.1.3.3 BIOFILM FORMATION

A biofilm is a complex network or "structure of microbes consortium supported with an extracellular matrix which attaches to the surface" of the host (Wibawa, 2016). *C. albicans* often forms biofilm on medical devices, such as catheters, and host cell surfaces (Baillie and Douglas, 1999, Nobile and Johnson, 2015). Both yeast and hyphal phases are essential for biofilm formation (Nobile and Johnson, 2015, Wibawa, 2016). Biofilm serves as a reservoir for a source of infections (Ramage et al., 2012, Wibawa, 2012, Wibawa, 2016). Persistent candidemia often results from *C. albicans* yeasts that are constantly released from biofilm (Kullberg and Arendrup, 2015). Biofilm also provides a protective physical barrier for yeast cells and helps them escape from immune surveillance and protection against physical and

chemical environment stresses (Chandra et al., 2007, Ramage et al., 2012, Wibawa, 2012, Wibawa, 2016).

1.1.3.4 PRODUCTION OF HYDROLYTIC ENZYMES

C. albicans produces various hydrolytic enzymes to aid its pathogenic activities, including adherence to host surfaces, invasion of host tissues and evasion of the host's antifungal responses. Secreted aspartyl proteinases, phospholipases and haemolysins are three keys hydrolytic enzymes of *C. albicans* (Wibawa, 2016).

Secreted aspartyl proteinases (Saps) have been shown to support *C. albicans* in facilitating host invasion (Naglik et al., 2003). They are active in all candidiasis stages and cases (Staniszewska et al., 2012). Saps can degrade vital proteins that help maintain host structure, such as collagen and mucin (Fisher et al., 2011, Naglik et al., 2003). Their activity has been shown to link with *C. albicans* virulence (Fisher et al., 2011, Naglik et al., 2003, Schaller et al., 2000). *C. albicans* strains lacking Saps are less destructive in murine models, and they appeared to be less successful at colonising the host than those with functional Saps (Fisher et al., 2011, Hube et al., 1997).

Phospholipases destabilise host membranes by hydrolysing glycerophospholipids, a major component of mammalian membranes (Fisher et al., 2011, Wilson et al., 2016). *C. albicans* isolates from candidemia patients appeared to have higher phospholipase activity than those obtained from oral cavities of healthy individuals, which reflected the virulence of these isolates (Ibrahim et al., 1995). Also, phospholipase-B knockout *C. albicans* mutants have shown to be less invasive than those with intact phospholipase-B (Leidich et al., 1998).

C. albicans produces **haemolysins** to induce haemolysis (rupturing of erythrocytes). This is important for *C. albicans* growth because erythrocytes contain haemoglobin, a protein that binds to iron. By causing haemolysis, *C. albicans* can extract its iron nutrient from haemoglobin (Almeida et al., 2009, Fisher et al., 2011, Wibawa, 2016).

1.1.3.5 CANDIDALYSIN

Candidalysin is a cytolytic peptide toxin secreted by *C. albicans* hyphae and was found to drive mucosal pathogenesis and modulate host immune responses by inducing epithelial activation and damage via MAPK signalling (Ho et al., 2019, Moyes et al., 2016, Swidergall et al., 2019a). Mutants that lack candidalysin could transition from yeast to hyphal phase with a similar invasion ability to the wild type control. However, they failed to induce epithelial damage and pro-inflammatory cytokine secretion (Moyes et al., 2016).

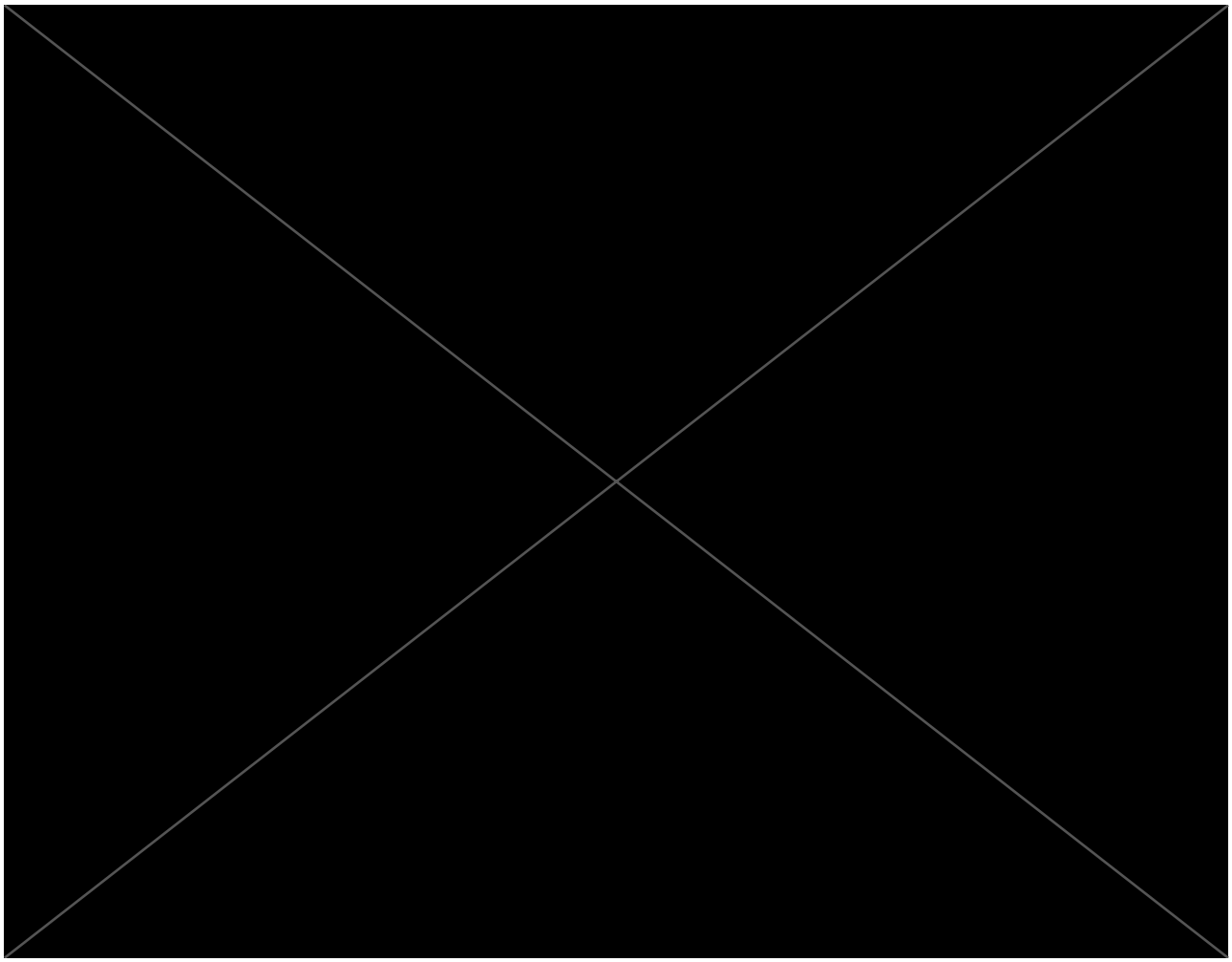
1.2 NEUTROPHILS ARE THE FIRST LINE OF DEFENCE AGAINST FUNGAL INVADERS

1.2.1 NEUTROPHILS ARE MYELOID POLYMORPHONUCLEAR CELLS

Neutrophils are a type of polymorphonuclear innate immune cell that act as a fast primary defence against fungal infections, such as candidiasis (Abbas et al., 2018, Gazendam et al., 2016). They are the most abundant white blood cells (WBCs or leukocytes), representing about 70% of all WBCs (Abbas et al., 2018, Amulic et al., 2012, Dancey et al., 1976, Gazendam et al., 2016). Neutrophils have one of the shortest lifespans of human cells: ~6-8 hours in circulation (Abbas et al., 2018, Amulic et al., 2012). However, their turnover is rapid, as around 100 billion cells are released from the bone marrow per day (Abbas et al., 2018, Amulic et al., 2012, Dancey et al., 1976, Scher et al., 2013, Summers et al., 2010).

Neutrophils are derived from haematopoietic stem cells (HSCs) and develop in the bone marrow during a process called myelopoiesis under the instruction of various growth factors and cytokines, such as granulocyte colony-stimulating factor (G-CSF) (Figure 1.5). Pluripotent HSCs first differentiate into granulocyte precursor cells (myeloblasts). These precursor cells then undergoing sequential differentiation, and approximately after 14 days, mature neutrophils are released into the circulation. During neutrophil differentiation, neutrophils form three distinct granule subsets that contain many essential antimicrobial proteins that are important for fungal infections: primary [or azurophilic, containing, for example, antimicrobial defensins, myeloperoxidase (MPO), lysozyme and Cathepsin G], secondary [or specific,

containing lysozyme and lactoferrin, for example] and tertiary [or gelatinase, containing gelatinase] granules (Abbas et al., 2018, Amulic et al., 2012, Borregaard, 2010, Borregaard and Cowland, 1997, Scher et al., 2013). During differentiation, neutrophils also develop a lobulated nucleus composed of transcriptionally inactive chromatin (Abbas et al., 2018, Amulic et al., 2012, Borregaard, 2010, Borregaard and Cowland, 1997, Scher et al., 2013).

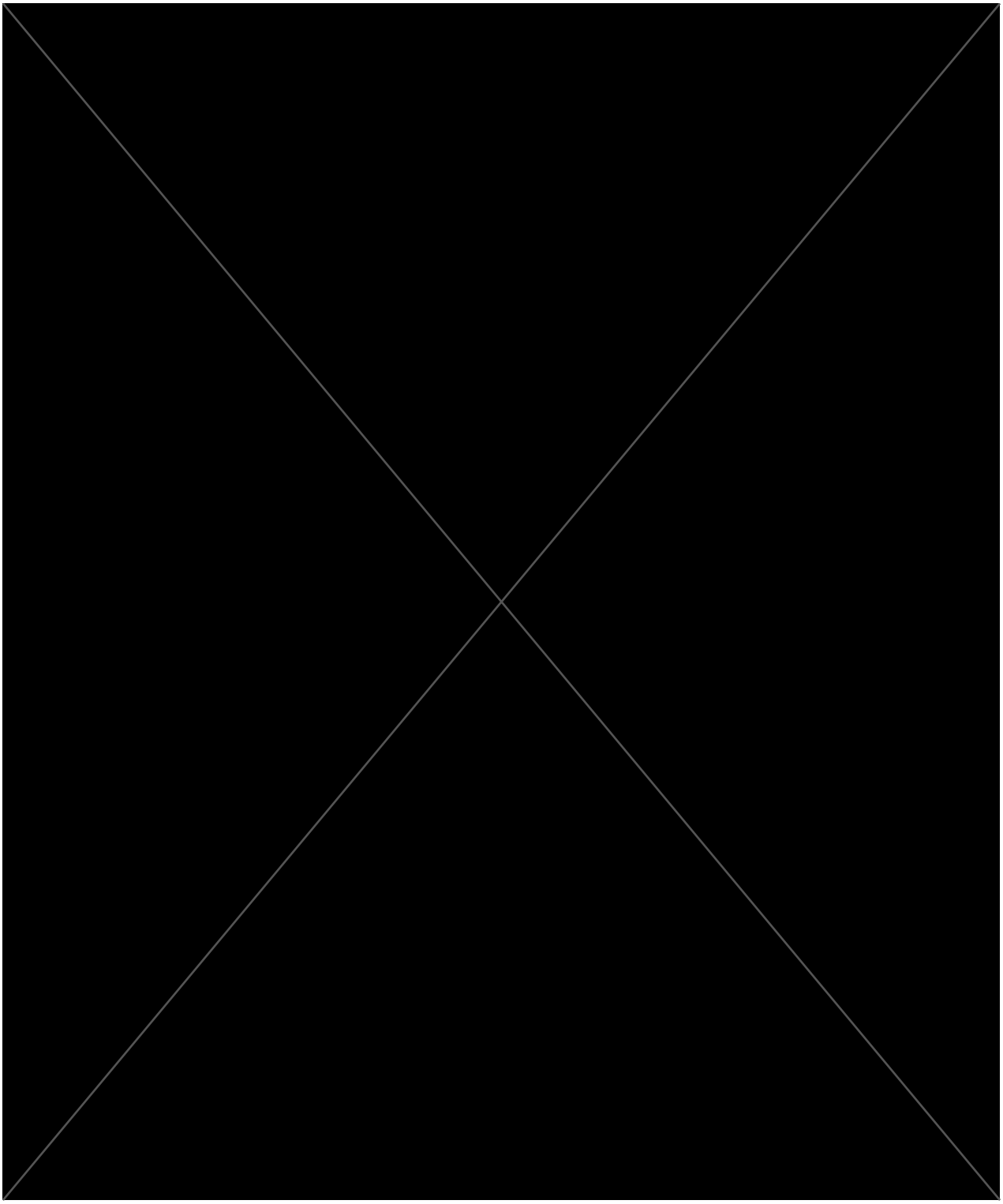


1.2.2 NEUTROPHILS ARE THE FIRST CELLS TO MIGRATE TO THE SITE OF AN INFECTION

During infection, neutrophils are the first immune cells to be recruited from circulation to the site of an infection, for example, in response to chemokines produced by tissue-resident macrophages. Although mononuclear macrophages are some of the first cells to interact with pathogens, they are nowhere near as potent as neutrophils, which can utilise several antimicrobial methods to kill pathogens, such as *C. albicans* (Abbas et al., 2018, Ermert et al., 2013, Rudkin et al., 2013, Vonk et al., 2002). The importance of neutrophil functions in candidiasis can be demonstrated by the increasing risk of candidemia in neutropenic patients (Pappas et al., 2016, Pfaller and Diekema, 2007).

1.2.2.1 NEUTROPHIL RECRUITMENT

During candidiasis (Figure 1.6), *C. albicans* induces tissue damage and inflammation, for example, by candidalysin, which results in the production of various pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6 and IL-17, as well as the production of G-CSF (Abbas et al., 2018, Amulic et al., 2012, Borregaard, 2010, Cheng et al., 2012, Moyes et al., 2016, Swidergall et al., 2019a). These cytokines cause the upregulation of endothelial adhesion molecules on the vascular endothelium surface, causing neutrophil tethering to vascular endothelium. Neutrophils then engage in transendothelial-migration through tight junctions via a process called diapedesis, moving to the site of infection down concentration gradients of either host-derived chemotactic cytokines, such as IL-8, or pathogen-derived chemoattractants, such as *C. albicans* β-1,3-D-glucans (Abbas et al., 2018, Amulic et al., 2012, Borregaard, 2010, Sato et al., 2006).



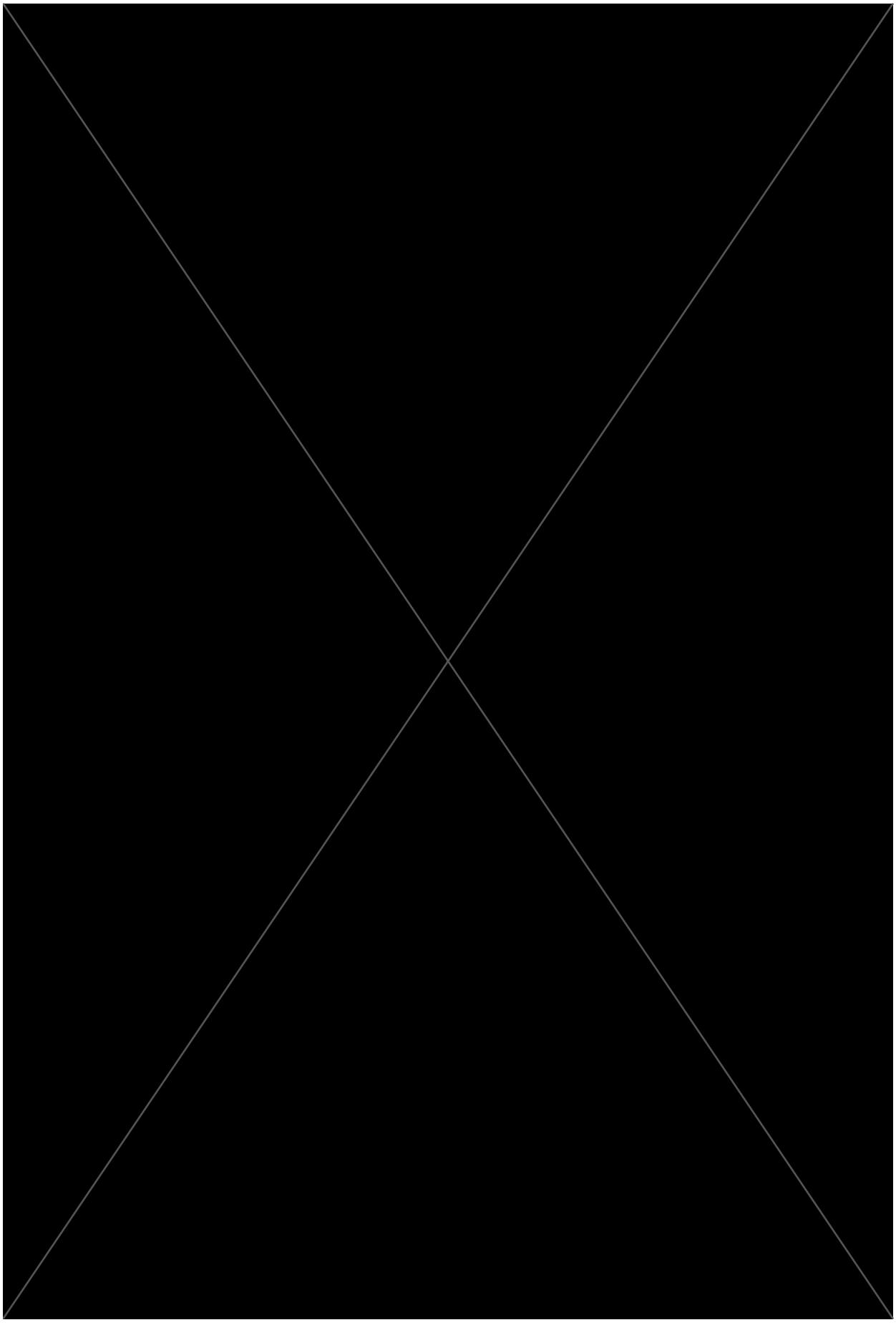
Patients with autosomal recessive leukocyte adhesion deficiency (LAD) syndromes have neutrophils that cannot exit blood circulation due to mutations in various receptors required for transmigration and thus cannot migrate to the site of infection (Etzioni, 1996, Etzioni, 2010,

von Andrian et al., 1993). Patients with LAD syndromes are often infected by *C. albicans* and die from fungal septicaemia (Movahedi et al., 2007, Parvaneh et al., 2010). To emphasise the importance of neutrophil recruitment, mouse studies also showed that neutrophils lacking integrins and mice lacking ICAMs have impaired migration and impaired ability to kill *C. albicans* (Davis et al., 1996, Soloviev et al., 2011).

1.2.2.2 PATHOGEN RECOGNITION RECEPTORS

The cell wall of *C. albicans* is essential for maintaining viability. *C. albicans* is composed of components (pathogen-associated molecular patterns or PAMPs) that are generally not present in our body, such as β-glucans, mannan and chitin (Gazendam et al., 2016, Netea et al., 2008). Neutrophils express various types of pattern recognition receptors (PRRs) to recognise several kinds of *C. albicans* PAMPs (Figure 1.7) (Gazendam et al., 2016).

One of the main neutrophil PRRs are **Toll-like receptors** (TLRs). TLRs consist of two main domains: an extracellular domain that is responsible for *C. albicans* structure recognition and a cytoplasmic domain that is responsible for inducing intracellular response (Abbas et al., 2018, Gow et al., 2012). Membrane-bound TLRs, such as TLR-2 and TLR-4, recognise phospholipomannans and O-linked mannans, respectively. Intracellular TLRs, such as TLR-9, recognise *C. albicans* DNA (Gow et al., 2012, Netea et al., 2008, Netea et al., 2002). Mice deficient in MyD88, the common downstream adaptor molecule for various TLRs, were reported to be susceptible to invasive candidiasis, thus demonstrating the significant global role of TLRs in defence against *C. albicans* infection (Marr et al., 2003, Netea et al., 2008, Villamón et al., 2004a). Further studies also reported that TLR-2 deficient mice are susceptible to disseminated candidiasis (Netea et al., 2006, Netea et al., 2002, Villamón et al., 2004b).



C-type lectin receptors (CLRs) are the most crucial type of receptors that neutrophils use to recognise *C. albicans* polysaccharide structures. Two main CLRs expressed on human neutrophils are dectin-1 (encoded by human *CLEC7A*) that recognises β-glucans (such as β-1,3-and β-1,6-glucans), and dectin-2 (encoded by human *CLEC6A*) that binds to α-mannans (Gazendam et al., 2016, Gow et al., 2012). The binding of dectin-1 or dectin-2 to their ligands initiates neutrophil intracellular signalling mediated through various pathways, such as Syk-CARD9 and NF-κB, to produce antifungal responses (Brown and Gordon, 2001, Gazendam et al., 2016, Gow et al., 2012). The vital role of dectin-1 and dectin-2 in anti-*C. albicans* defence can be demonstrated by *in vivo* studies, whereby dectin-1 and dectin-2 knockout mice have an increased susceptibility to systemic candidiasis (Brown and Gordon, 2001, Gazendam et al., 2016, Saijo et al., 2010, Taylor et al., 2007). Patients with CARD9 deficiency are vulnerable to *C. albicans* infections, and neutrophils isolated from these patients were defective in *C. albicans* killing (Drewniak et al., 2013, Drummond et al., 2019, Glocker et al., 2009).

There are many other PRRs that neutrophils use to recognise *C. albicans,* such as **complement receptor (CR)-3 integrins** that recognise β-glucans and **Fc-gamma receptors** (FcγRs) that bind to antibodies of opsonised *C. albicans* (Gazendam et al., 2016). All of these PRRs synergistically cooperate to promote pro-inflammatory and antifungal responses.

1.2.3 NEUTROPHIL ANTIFUNGAL RESPONSES

Neutrophils become activated upon the binding of their PRRs to *C. albicans* PAMPs. They then utilise many antifungal mechanisms to counteract *C. albicans*, including cytokine production, phagocytosis, degranulation and neutrophil extracellular traps (NETs) (Amulic et al., 2012, Gazendam et al., 2016).

1.2.3.1 CYTOKINE PRODUCTION

Cytokine production is an indirect strategy of neutrophils to suppress *C. albicans.* When neutrophils encounter *C. albicans*, they release various cytokines and chemokines to attract and crosstalk with other immune cells (Amulic et al., 2012, Niemiec et al., 2017, Scapani et al., 2000). A transcriptional study showed an increase in cytokine genes of neutrophils in response to *C. albicans*, such as those encoding for macrophage inflammatory protein (MIP)-1β, IL-1β, IL-8, macrophage-colony stimulating factor (M-CSF) and TNF-α (Niemiec et al., 2017). IL-8 is responsible for recruiting other neutrophils, while IL-1β and TNF-α induce other local cells to produce neutrophil chemoattractants (Amulic et al., 2012, Scapani et al., 2000). Other cytokines produced by neutrophils, such as MIP-1α and IL-12, are important for recruitment of monocytes, macrophages, natural killer (NK) cells and dendritic cells, and induction of Th1 lymphocyte differentiation and responses (Abbas et al., 2018, Amulic et al., 2012, Leonard and Yoshima, 1990, Tateda et al., 2001).

1.2.3.2 PHAGOCYTOSIS

Phagocytosis is a key process in the neutrophil antifungal response against *C. albicans* (Gazendam et al., 2016). Phagocytosis is initiated when neutrophil PRRs bind to *C. albicans* PAMPs or opsonised *C. albicans* (Amulic et al., 2012, Gazendam et al., 2016). First, the

neutrophil engulfs or phagocytoses *C. albicans* by surrounding its pseudopodium around the yeast cell, forming a phagosome. The phagosome then fuses with neutrophil preformed granules, containing several enzymes and antimicrobial proteins that mediate *C. albicans* killing (Amulic et al., 2012, Segal, 2005). Ultimately, *C. albicans* gets digested and killed by neutrophils by enzymatic killing and toxic reactive oxygen species. Once neutrophils have performed their antifungal function, they die via apoptosis and get cleared by macrophages (Amulic et al., 2012, Abbas et al., 2018).

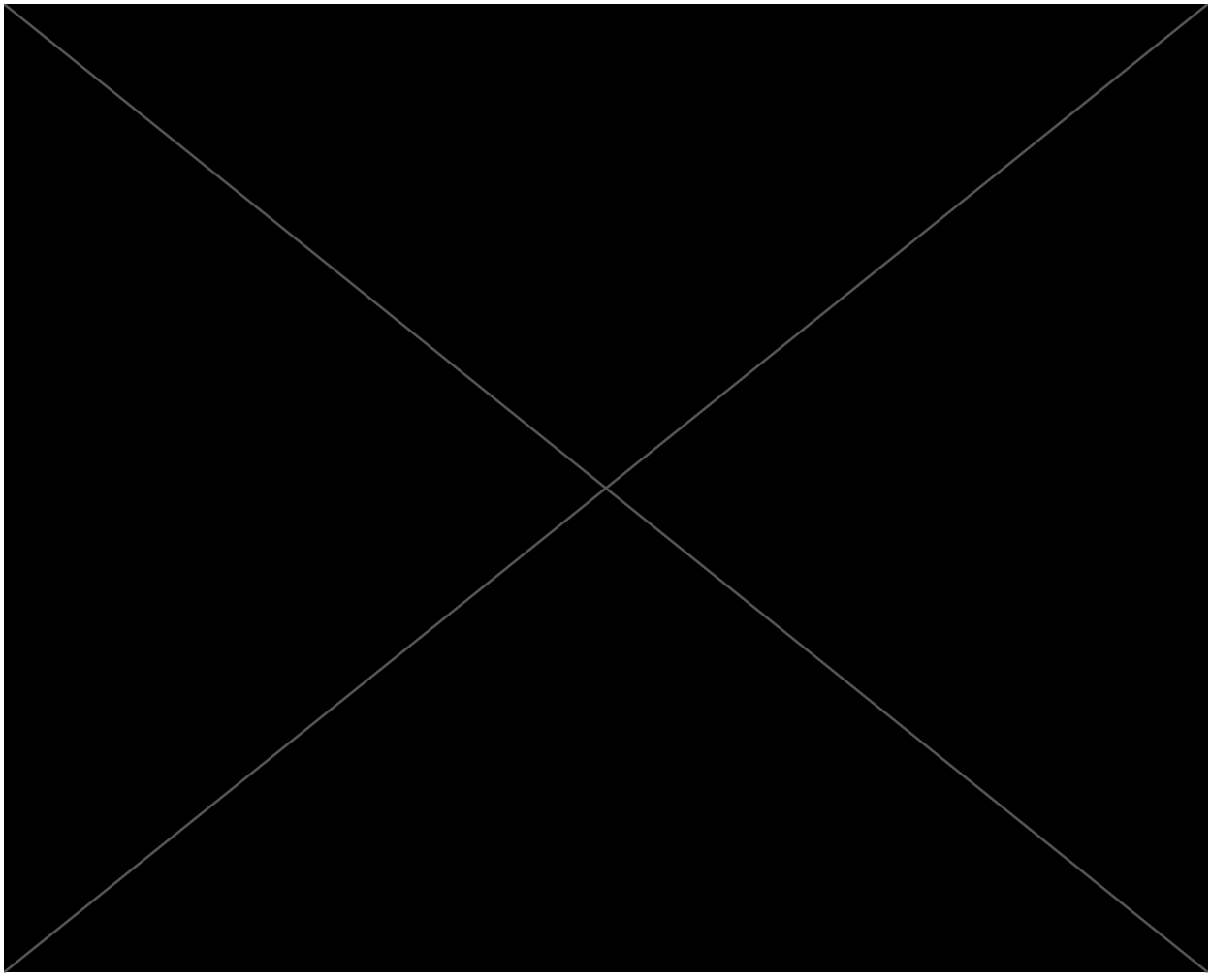
1.2.3.3 DEGRANULATION

Degranulation is a process where a neutrophil releases its antimicrobial factors and enzymes and other molecules from its "granules" or secretory vesicles (Lacy, 2006). Neutrophil granules can either fuse into the phagosome to kill pathogens or fuse with the plasma membrane and degranulate extracellularly via a process called exocytosis (Amulic et al., 2012). Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is an autosomal recessive disorder caused by mutations in *STXBP2*, a gene encoding for syntaxin binding protein Munc18-2 required for the fusion of granules to the plasma or phagosomal membrane (zur Stadt et al., 2009). FHL-5 patients are deficient in Munc18-2 proteins, and their neutrophils were found to be defective at killing *C. albicans*, thus demonstrating the importance of neutrophil degranulation (Gazendam et al., 2016).

1.2.3.4 OXIDATIVE RESPIRATORY BURST

Oxidative respiratory burst is one of the main antifungal mechanisms of neutrophils (Figure 1.8). This process is assisted by the fusion of specific granules to the plasma membrane or phagosome. Specific granules contain flavocytochrome b558, a catalytic core that is vital for NADPH oxidase (NOX2) complex activation and reactive oxygen species (ROS) production,

such as superoxide anions (O_2^{-}) and hydrogen peroxide (H_2O_2) (Amulic et al., 2012, Segal, 2005). Similarly, when azurophilic granules fuse with plasma or phagosomal membrane, they release MPO - an enzyme that converts hydrogen peroxide into other reactive species, such as hypochlorous acid (HOCL) (Amulic et al., 2012, Jamieson et al., 1996, Segal, 2005). ROS are highly unstable molecules that can react with and modify various organic substances, such as proteins and DNA of *C. albicans*, leading to candidal cell death (Amulic et al., 2012, Jamieson et al., 1996, Kobayashi et al., 2002, Segal, 2005). A defect in any components of NOX2 often result in the loss of ROS production and thus impaired neutrophil antifungal responses (Amulic et al., 2012, Gazendam et al., 2016). For example, patients with chronic granulomatous disease, an immunodeficiency caused by mutations in NOX2 complex genes, often suffer from recurrent *C. albicans* infections (Cohen et al., 1981, Gazendam et al., 2016, Song et al., 2011).

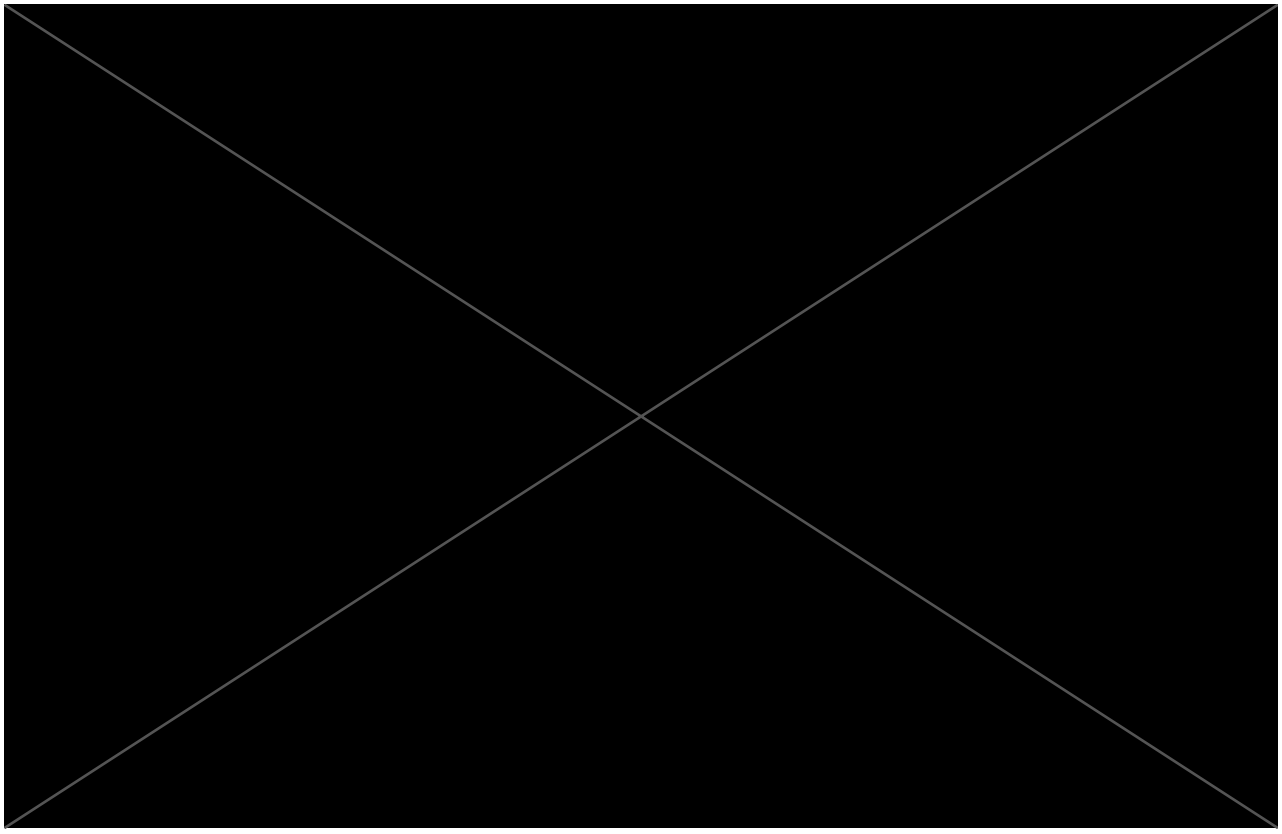


In addition to NOX2 components, neutrophil granules contain other enzymes and antimicrobial proteins, which are important in *C. albicans* killing. For example, lysozyme or muramidase (stored in both azurophilic and specific granules) is vital in breaking down *C. albicans* cell wall components (Amulic et al., 2012, Marquis et al., 1991, Marquis et al., 1982). It was also found to degrade *Candida* Saps and thus inhibited the growth of *C. albicans* (Wu et al., 1999). Cathepsin G, neutrophil elastase and proteinase 3, from azurophilic granules, act similarly by cleaving fungal proteins via proteolysis (Amulic et al., 2012, Faurschou and Borregaard, 2003, Segal, 2005). Proteinase 3 was found to cleave the C-terminus of hCAP-18 (human cathelicidin) to release LL-37 (Sørensen et al., 2001). LL-37 is an antimicrobial peptide that has been shown to enhance the β-1,3-exoglucanase activity of Xog1p, which reduces *C. albicans* cell wall integrity (Chang et al., 2012). Azurophilic granules also contain antimicrobial defensins, such as α-and β-defensins (Amulic et al., 2012). Human neutrophil α-defensin-1 was found to kill *C. albicans* by inducing ATP efflux and depleting intracellular ATP levels (Edgerton et al., 2000). Human β-defensin-3 was found to reduce *C. albicans* adherence ability and its viability by disrupting fungal cell wall formation (Chang et al., 2012, Feng et al., 2005).

Similar to azurophilic granules, specific granules also contain antimicrobial proteins that are involved in *C. albicans* killing. For example, lactoferrin was shown to inhibit the growth of *C. albicans* by binding to its iron nutrient (Bellamy et al., 1993, Kirkpatrick et al., 1971). Another example is PTX3, a soluble PRR that can enhance *C. albicans* clearance by increasing phagocytotic uptake through the interaction with complement factors and bringing NET antimicrobial factors into proximity with the fungus (Daigo and Hamakubo, 2012, Ma et al., 2011).

1.2.3.5 NEUTROPHIL EXTRACELLULAR TRAPS

The production of neutrophil extracellular traps (NETs) is another crucial microbe killing mechanism. NETs are characterised by large, extracellular, web-like structures of DNA, histones and granule antimicrobial proteins (Figure 1.9). They also contain molecules that mediate and activate immune responses, such as alarmins (Amulic and Sollberger, 2019, Branzk et al., 2014, Papayannopoulos, 2018). When neutrophils come across pathogens that are too large to phagocytose, they undergo a unique form of death, called NETosis, whereby NETs are released and entrap pathogens (Branzk et al., 2014). This is particularly important as an antifungal response against *C. albicans*, a species that can switch its phenotype from a small yeast cell to a large filamentous hypha (Pappas et al., 2018). Studies have demonstrated that neutrophils release NETs as an antifungal response to *C. albicans*, with a predominant effect on those in hyphal form (Branzk et al., 2014, Urban et al., 2006). Also, the neutrophil dectin-2 receptor was found to mediate NETosis upon binding *C. albicans* (Miura et al., 2019, Papayannopoulos, 2018, Wu et al., 2019). Furthermore, a study has revealed that calprotectin, a component of NETs, plays a vital role in the clearance of *C. albicans* infections (Urban et al., 2009). Calprotectin is a cytosolic protein complex that interferes with *C. albicans* growth and survival by chelating their essential metal ion nutrients, such as Zn^{2+} and Cu^{2+} (Besold et al., 2018, Sohn and Hahn, 1996).



1.3 *CANDIDA ALBICANS* EVOLVED EFFECTIVE STRATEGIES TO ESCAPE AND EVADE NEUTROPHIL ANTIFUNGAL RESPONSES

1.3.1 *C. ALBICANS* UTILISES ITS VIRULENCE FACTORS TO ESCAPE NEUTROPHIL KILLING

To survive and reproduce, *C. albicans* has developed novel strategies to escape from being killed by neutrophils. Its virulence factors can help it to achieve this by interfering with neutrophil antifungal responses. These strategies include the following:

1.3.1.1 DIMORPHISM

Each *C. albicans* morphology has its own advantage in terms of escape from neutrophil antifungal responses. For example, the hyphal phase was found to be resistant to phagocytosis, while the yeast phase was found to induce less pro-inflammatory cytokines and neutrophil chemoattractants from epithelial cells (Jayatilake et al., 2007, Moyes et al., 2010, Naglik et al., 2011, Salvatori et al., 2018). A mouse study also showed that engulfed *C. albicans* yeast could escape from the neutrophil phagosome by switching to hyphal growth and causing the cell to rupture (Ermert et al., 2013). Therefore, the ability to undergo yeast-hyphae morphogenesis is vital in helping *C. albicans* to escape neutrophil killing.

1.3.1.2 BIOFILM FORMATION

Biofilm provides a protective physical barrier for yeast cells and helps them escape from neutrophil surveillance (Wibawa, 2012). Biofilm formation of *C. albicans* has been shown to

reduce neutrophil-mediated killing by preventing phagocytosis, ROS and NETs release (Johnson et al., 2016, Kernien et al., 2020, Lohse et al., 2018, Xie et al., 2012). Therefore, the biofilm formation ability of *C. albicans* serves as a crucial strategy in its neutrophil resistance.

1.3.1.3 PRODUCTION OF HYDROLYTIC ENZYMES

Hydrolytic enzymes, such as Saps, were found to have the ability to degrade C5a complement (an important molecule for neutrophil recruitment and activation), as well as other complement proteins that prime *C. albicans* for neutrophil recognition and killing (Abbas et al., 2018, Gazendam et al., 2016, Singh et al., 2020). They were also found to degrade IL-37. IL-37 is important for ROS and IL-8 production, and it has the ability to prolong neutrophil lifespan (Rapala-Kozik et al., 2015). It is unclear how phospholipases and haemolysins interfere with neutrophil killing, but evidence suggested that they are important in *C. albicans* survival (Fisher et al., 2011, Wibawa, 2016).

1.3.1.4 ANTIOXIDANT PROTEINS

C. albicans contains superoxide dismutases (Sods), enzymes that detoxify ROS from neutrophils. Sods eliminate superoxide anions by catalysing the reaction that converts $O_2^{\cdot -}$ into a less reactive H_2O_2 species (Martchenko et al., 2004, Poulain, 2015). There are six Sod proteins. Some of them were associated with the fungal cell wall, such as Sod4, Sod5 and Sod6, that provide a quicker response to neutrophil antifungal responses than those in the cytoplasm (Fradin et al., 2005, Niemiec et al., 2017, Poulain, 2015). *SOD* genes were also found to be upregulated in yeast cells when interacting with neutrophils (Niemiec et al., 2017). The importance of Sod proteins can be further demonstrated by the sensitivity and susceptibility of mutants lacking Sod enzymes, such as Sod5, to neutrophil killing (Fradin et al., 2005).

1.3.1.5 ALTERING CELLULAR METABOLISM

C. albicans can alter its metabolism as part of a stress response when encountering neutrophils. *C. albicans* was found to upregulate the glyoxylate cycle upon phagocytosis (Barelle et al., 2006, Miramon et al., 2012). For example, *C. albicans* was found to upregulate *ICL1* (encodes for isocitrate) and *MLS1* (encodes for malate synthase) which are vital in the activation of the glyoxylate cycle (Barelle et al., 2006, Lorenz and Fink, 2001). *C. albicans* lacking *ICL1* was found to be more vulnerable to neutrophil killing (Miramon et al., 2012). This adaptation of *C. albicans* is crucial for its survival in an environment where nutrients and oxygen supplies are restricted, such as in the phagosome (Barelle et al., 2006).

1.3.1.6 INDUCTION OF STRESS RESPONSE PATHWAYS

C. albicans was found to activate MAPK pathways, such as Hog1, Cek1 and Ras1-GTPase, to resist neutrophil defences (Alonso-Monge et al., 2003, Csank et al., 1998, Ernst and Pla, 2011, Hollomon et al., 2016, Miramon et al., 2012, Salvatori et al., 2018). The Hog1 pathway is crucial in *C. albicans* resistance to osmotic and oxidative stresses, while the Cek1 pathway is vital for *C. albicans* cell wall biogenesis and morphogenesis (Alonso-Monge et al., 2003, Csank et al., 1998, Ernst and Pla, 2011, Miramon et al., 2012). Similarly, the Ras1-GTPase pathway is also important in *C. albicans* morphogenesis and its responses against pH stress (Hollomon et al., 2016, Salvatori et al., 2018). Hog1 or Cek1 deletion mutants were shown to be more susceptible to neutrophil killing, while deletion of Ras1 was shown to have an opposite effect (Miramon et al., 2012, Salvatori et al., 2018).

There are many more mechanisms that *C. albicans* utilises to counteract neutrophil antifungal responses. There will also likely be more *C. albicans* genes to be involved in these anti-

neutrophil-mediated killing responses. By identifying these novel genes, it could be possible to propose new therapeutic targets for *C. albicans* infections, such as candidemia.

1.4 AIMS AND OBJECTIVES

This research project aimed to identify *C. albicans* genetic factors that are important for the survival of neutrophil antifungal responses by screening the GRACE™ mutant library.

The main research objectives were:

1. To optimise a high throughput chemiluminescence-based killing assay using alamarBlue™ and Calcofluor White reagents
2. To establish a suitable multiplicity of infection (MOI) of *C. albicans* for the killing assay
3. To establish PLB-985 cells (neutrophil-like cells) as a model for *C. albicans* killing assay
4. To identify *C. albicans* mutants from the GRACE™ library that are susceptible to PLB-985 killing
5. To validate *C. albicans* mutants from the GRACE™ library that are susceptible to PLB-985 killing with neutrophils
6. To characterise *C. albicans* mutants (that are susceptible to neutrophils), using the following assays:
    - Ability to form somatic hyphae
    - Susceptibility to hydrogen peroxide
    - Susceptibility to cell wall stressors
    - Ability to induce neutrophil ROS
    - Ability to induce NETs

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 *CANDIDA ALBICANS* STRAINS USED IN THIS STUDY

Strains	Source	Reference
CaSS1, Wild-type (WT) - GRACE™ library background strain	Merck & Co.	(Roemer et al., 2003)
GRACE™ strain library	Merck & Co.	(Roemer et al., 2003)
CAI4	Bernhard Hube	(Fradin et al., 2005)
CAI4 - *efg1Δ/Δ cph1Δ/Δ*	Bernhard Hube	(Wartenberg et al., 2014)
SN95	Leah Cowen	(Noble and Johnson, 2005)
SN95 - *hog1Δ/Δ*	Carolyn Williamson	Diezmann lab archive
SN95 - *tup1Δ/Δ*	Michelle Leach	Diezmann lab archive
SN95 - *mkk2Δ/Δ*	Carolyn Williamson	Diezmann lab archive
SN95 - *pkc1Δ/Δ*	Carolyn Williamson	Diezmann lab archive

2.1.2 EUKARYOTIC CELLS USED IN THIS STUDY

Primary human neutrophils	Extracted from healthy donor blood
Promyeloblast leukaemia HL-60 (PLB-985)	Kind gift from Dr. Mary Dinauer

2.1.3 *C. ALBICANS* CULTURE MEDIA & ADDITIVES

D-Glucose (Anhydrous)	Melford Biolaboratories Limited
Doxycycline hydrochloride (as hyclate)	Duchefa Biochemie B.V.
Peptone	Sigma-Aldrich
Yeast extract (YE)	Sigma-Aldrich

2.1.4 EUKARYOTIC CELL CULTURE & DIFFERENTIATION MEDIA AND ADDITIVES

Clear RPMI-1640 medium (without Q, without phenol red)	Gibco, Thermo Fisher Scientific
Foetal bovine serum (FBS/FCS)	Gibco, Thermo Fisher Scientific
L-Glutamine (Q)	Gibco, Thermo Fisher Scientific
N, N-Dimethylformamide (DMF)	Sigma-Aldrich
Nutridoma-CS	Roche, Sigma-Aldrich
Penicillin/Streptomycin/Glutamine (P/S/Q)	Gibco, Thermo Fisher Scientific
Red RPMI-1640 medium (without Q, with phenol red)	Gibco, Thermo Fisher Scientific

2.1.5 PLB-985 & PRIMARY HUMAN NEUTROPHIL ISOLATION

EasySep™ Direct Human Neutrophil Isolation Cocktail	Stemcell™ Technologies
EasySep™ Direct RapidSpheres™ 50300	Stemcell™ Technologies
EasySep™ Human Neutrophil Isolation Kit	Stemcell™ Technologies

Ethylenediaminetetraacetic acid (EDTA)	Made by media kitchen from the University of Bristol
Histopaque 1077	Sigma-Aldrich
Histopaque 1119	Sigma-Aldrich
Human Serum Albumin (HSA)	Seqens
Percoll™	GE Healthcare
Phosphate-buffered saline (PBS), pH7.4	Gibco, Thermo Fisher Scientific

2.1.6 *C. ALBICANS* – NEUTROPHIL KILLING ASSAY

alamarBlue™ Cell Viability Reagent	Thermo Fisher Scientific
Calcofluor White Stain 18909 (CFW)	Sigma-Aldrich
Clear RPMI-1640 medium (without Q, without phenol red)	Gibco, Thermo Fisher Scientific
Doxycycline hydrochloride (as hyclate)	Duchefa Biochemie B.V.
Human pooled plasma serum	Extracted from healthy donor blood
Paraformaldehyde (PFA) 4% in PBS	VWR
PBS tablets	Oxoid
Penicillin-Streptomycin-Glutamine (P/S/Q)	Gibco, Thermo Fisher Scientific
Triton X-100	Thermo Fisher Scientific

2.1.7 *C. ALBICANS* SERUM-INDUCED FILAMENTATION ASSAY

FBS	Gibco, Thermo Fisher Scientific
PBS tablets	Oxoid

2.1.8 *C. ALBICANS* OXIDATIVE STRESS ASSAY

Agar	Sigma-Aldrich
D-Glucose (Anhydrous)	Melford Biolaboratories Limited
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich
PBS tablets	Oxoid
Peptone	Sigma-Aldrich
Yeast extract (YE)	Sigma-Aldrich

2.1.9 *C. ALBICANS* CELL WALL STRESS ASSAY

Agar	Sigma-Aldrich
Calcofluor White Stain 18909 (CFW)	Sigma-Aldrich
Congo Red	Sigma-Aldrich
D-Glucose (Anhydrous)	Melford Biolaboratories Limited
M 2-(*N*-morpholine)-ethane sulfonic acid-sodium hydroxide (MES-NaOH) pH 6.0	Sigma-Aldrich
Peptone	Sigma-Aldrich
YE	Sigma-Aldrich

2.1.10 OXIDATIVE BURST ASSAY

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Gibco, Thermo Fisher Scientific
Hank's Balanced Salt Solution (HBSS) (with Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions)	Lonza

Horseradish peroxidase (HRP)	Sigma-Aldrich
Luminol	Sigma-Aldrich
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich

2.1.11 NETOSIS ASSAY

Clear RPMI-1640 medium (without Q, without phenol red)	Gibco, Thermo Fisher Scientific
Doxycycline hydrochloride (as hyclate)	Duchefa Biochemie B.V.
HEPES	Gibco, Thermo Fisher Scientific
HSA	Lonza
Human pooled plasma serum	Extracted from healthy donor blood
SYTO™ Green	Thermo Fisher Scientific
SYTOX™ Orange	Thermo Fisher Scientific

2.2 METHODS

2.2.1 MULTIPLICITY OF INFECTION (MOI) EQUATIONS

2.2.1.1 MOI EQUATION OF OD_{600} USING EPPENDORF BIOSPECTROMETER® BASIC

$$*OD_{600} \text{ of } 1 = 2 \times 10^7 \text{ cell/mL}$$

2.2.1.2 MOI EQUATION OF OD_{595} USING MULTISKAN™ FC MICROPLATE PHOTOMETER (THERMO FISHER SCIENTIFIC)

$$*OD_{595} \text{ of } 1 = 1 \times 10^7 \text{ cell/mL}$$

*The optical density (OD) of *C. albicans* was measured at a wavelength of 595 or 600 nm to estimate the concentration of *C. albicans* cells in suspension. These equations were previously established by the Diezmann laboratory. They derived from the standard curve of $OD_{595/600}$ against colony forming units (CFU)/mL.

2.2.2 *C. ALBICANS* CELL CULTURE

2.2.2.1 CULTURING OF *C. ALBICANS* IN CULTURE TUBES

Overnight: Wild-type (WT) strain or GRACE™ mutants were inoculated into culture tubes with 10 mL of YPD (2% D-glucose, 2% peptone, 1% YE) media (with 0.05 µg/mL of doxycycline**) and incubated overnight for 17 hours at 30 °C and 200 rpm.

Subculture: Each overnight culture was diluted with YPD media (with 0.05 µg/mL of doxycycline) accordingly to get OD_{600} of 1. 50 mL of each diluted culture was subcultured for 3 hours at 30 °C and 200 rpm.

**The GRACE™ mutants were created by deleting one allele of a target gene and placing the other copy under the control of a Tetracycline (Tet) repressive promoter. Doxycycline, a Tet analogue, is added to the culture to inhibit target gene expression completely (Roemer et al., 2003).

2.2.2.2 OPTIMISATION: CULTURING OF *C. ALBICANS* IN A 96-WELL PLATE

Overnight: WT strain was inoculated into a clear flat-bottom 96-well plate (Corning Incorporated) filled with 100 µL of YPD media, using a 96 long-pin RePad (Singer). The plate was sealed with breathable rayon film (VWR) and incubated overnight for 17 hours at 30 °C and 800 rpm.

Subculture: The overnight WT cultures were diluted with YPD media, using the following dilution factors: 1, ½, ¼. A 96 floating E-Clip style (VP408F6S.5, V & P Scientific) was used to transfer the diluted cultures into a clear flat-bottom 96-well plate (Corning incorporated) filled with 100 µL of YPD media. The plate was sealed with breathable rayon film (VWR) and incubated for 3 hours at 30 °C and 800 rpm. After the incubation, the plate was centrifuged at 1000 x g for 3 minutes. The supernatant was removed, and cells were resuspended in 100 µL of 1X (w/v) PBS.

The plate OD_{595} values were used to determine the required volume of *C. albicans* suspension to obtain 150,000 cells/well for the GRACE™ library screen.

2.2.2.3 CULTURING OF THE GRACE™ LIBRARY PLATES

Overnight: WT strain and GRACE™ mutants were inoculated into clear flat-bottom 96-well plates (Corning incorporated) filled with 100 µL of YPD media (with 0.05 µg/mL of doxycycline), using a 96 long-pin RePad (Singer). These plates were sealed with breathable rayon film (VWR) and incubated overnight for 17 hours at 30 °C and 800 rpm.

Subculture: A 96 floating E-Clip style (VP408F6S.5, V & P Scientific) was used to transfer WT strain and GRACE™ mutants' overnight cultures into clear flat-bottom 96-well plates (Corning incorporated) filled with 100 µL of YPD media (with 0.05 µg/mL of doxycycline). These plates were sealed with breathable rayon film (VWR) and incubated for 3 hours at 30 °C and 800 rpm. After the incubation, the plates were centrifuged at 1000 x g for 3 minutes. The supernatant was removed, and cells were resuspended in 100 µL of clear RPMI-1490 with 1X (v/v) Penicillin/Streptomycin/Glutamine (P/S/Q), 6% human pooled plasma and 0.15 µL/mL of doxycycline.

2.2.2.4 *C. ALBICANS* GROWTH CURVE

C. albicans overnight cultures were diluted in YPD (with 0.05 µg/mL of doxycycline) to a starting OD_{595} of 0.02. 100 µL of each diluted culture was transferred into a clear flat-bottom 96-well plate (Corning incorporated). The plate was sealed with breathable rayon film (VWR), and OD_{595} was read every 15 minutes for 24 hours at 30 °C and shaking. Then, the growth rate (OD_{595} per hour) and maximum growth (OD_{595}) of each mutant were determined.

2.2.3 PLB-985 CELL CULTURE

2.2.3.1 PLB-985 CELL CULTURE

PLB-985 cells were cultured and maintained in a 25 cm^2 culture flask (Corning Incorporated) in culture media [red RPMI-1640 with 1X (v/v) P/Q/S, 10% (v/v) FBS] at 37 °C and 5% CO_2. To split the cells, the flask content was centrifuged at 450 x g for 5 minutes. The supernatant was aspirated, and the cell pellet was resuspended in either fresh culture media for maintenance or differentiation media for further investigations.

2.2.3.2 DIFFERENTIATION AND PURIFICATION OF PLB-985

$1x10^6$ PLB-985 cells were seeded in 3 mL of differentiation media [red RPMI-1640 with 1X (v/v) P/S/Q, 2.5% (v/v) FBS, 0.5% (v/v) DMF, 1X (v/v) Nutridoma-CS] per well in a 6-well plate (Corning Incorporated), and incubated at 37 °C and 5% CO_2 for 6 days. On day 4 of differentiation, each well was topped up with 2 mL of differentiation media. Cells were harvested on day 6 of differentiation. Cells were centrifuged at 400 x g for 10 minutes. The supernatant was discarded, and the concentrated cell pellet was resuspended in 1 mL of assay media [clear RPMI-1640 with 1% (v/v) FBS, 0.5% (v/v) DMF, 2 mM Q]. Then, cells were layered on top of 3 mL of Histopaque 1077 and centrifuged at 800 x g for 20 minutes. Differentiated PLB-985 cells floating on top of Histopaque 1077 were collected and washed with 3 mL of assay media and centrifuged at 400 x g for 10 minutes. The supernatant was aspirated, and the cell pellet was resuspended in 1 mL of 1X (w/v) PBS. Cells were then counted and ready for further experiments.

2.2.4 PRIMARY HUMAN NEUTROPHIL ISOLATION

2.2.4.1 ETHICAL STATEMENT

Blood samples from healthy volunteers were collected by trained staff from the Bristol Platelet Group (Poole and Hers laboratories). Human blood collection was approved by an NHS Research Ethics Committee, and all donors provided written informed consent.

2.2.4.2 NEUTROPHIL ISOLATION USING DENSITY GRADIENT METHOD

Blood samples from healthy donors were overlayed on top of Histopaque 1119 in a 1:1 ratio and centrifuged at 800 x g for 20 minutes. Neutrophil's layer was collected and washed with 0.2% HSA in 1X (w/v) PBS in a 1:2 ratio and centrifuged at 400 x g for 10 minutes. The supernatant was then aspirated. The cell pellet was resuspended in 1 mL of 0.2% HSA in 1X (w/v) PBS, layered on top of Percoll™ gradients (from 85% to 65%) and centrifuged at 800 x g for 20 minutes. Neutrophil's layer was collected and washed with 0.2% HSA in 1X (w/v) PBS in a 1:2 ratio and centrifuged at 400 x g for 10 minutes. The supernatant was aspirated. Neutrophil's pellet was resuspended in 0.2% HSA in 1X (w/v) PBS, ready for subsequent experiments.

2.2.4.3 NEUTROPHIL ISOLATION USING THE EASYSEP™ MAGNET NEGATIVE SELECTION METHOD (STEMCELL™ TECHNOLOGIES)

50 µL/mL of EasySep™ Direct Human Isolation Cocktail and EasySep™ Direct RapidSphere™ 50300 were mixed with blood samples from healthy donors in a 14 mL Falcon® polystyrene round-bottom tube (Thermo Scientific) and incubated for 5 minutes at room temperature. The sample tube was topped up and mixed with magnetic separation media [1 mM of EDTA in 1X (w/v) PBS, free from Ca^{2+} and Mg^{2+}] to either 10 mL for samples < 4 mL

or 12 mL for samples ≥ 4 mL. The tube was then placed into the EasySep™ magnet and incubated for 10 minutes at room temperature. The enriched neutrophil suspension was collected into a new tube, mixed with the same volume of EasySep™ Direct RapidSphere™ 50300 and incubated for 5 minutes at room temperature. The new tube was placed into the magnet and incubated for 5 minutes at room temperature for the second separation. The enriched neutrophil suspension was collected into a new tube and placed into the magnet for another 5 minutes for the final separation. The enriched neutrophil suspension was collected and centrifuged at 400 x g for 10 minutes. The supernatant was aspirated. Neutrophil's pellet was resuspended in 0.2% HSA in 1X (w/v) PBS, ready for subsequent experiments.

2.2.5 *C. ALBICANS* – NEUTROPHIL KILLING ASSAYS

2.2.5.1 OPTIMISATION: CALCOFLUOR WHITE VS. ALAMARBLUE™ STAINING

The WT overnight culture was diluted with 1X (w/v) PBS to obtain either 3 x 10^4 cells/well, 1.5 x 10^5 cells/well or 3 x 10^5 cells/well, using Eppendorf BioSpectrometer® basic. Each diluted culture was split into two tubes with equal volumes, where one tube was heat-killed by incubating in the water bath at 65°C for 30 minutes. Then, 90 µL of cell suspension of each culture was added into the wells of a black 96-well plate (Corning incorporated). 10 µL of either 10X (w/v) Calcofluor White (CFW) or 10X (w/v) alamarBlue™ was added into the appropriate wells. Using the FLUOstar Omega Microplate Reader (BMG LABTECH), fluorescence was recorded for 30 minutes in 5 minutes intervals for CFW (360/460 nm, 500 gains) and 24 hours in 2 hours intervals for alamarBlue™ (544/590 nm, 1000 gains).

2.2.5.2 OPTIMISATION: WILD TYPE KILLING ASSAY

50 µL of WT cell suspension [in clear RPMI-1640 with 1X (v/v) P/S/Q, 6% human pooled plasma and 0.15 µg/mL of doxycycline], of either MOI of 1 (3 x 10^4 cells), MOI of 5 (1.5 x 10^5 cells) or MOI of 10 (3 x 10^5 cells), was added to the wells of a black 96-well plate (Corning Incorporated). 100 µL of neutrophils or PLB-985 suspension (3 x 10^5 cell/mL in RPMI-1640) was added to the wells of the black 96-well plate. 100 µL of RPMI-1640 was added to the wells without neutrophils or PLB-985. The plate was then incubated at 37°C and 5% CO_2 for 2.5 hours. After incubation, either CFW or alamarBlue™ assays were carried out (see section 2.2.5.5-6).

2.2.5.3 GRACE™ LIBRARY SCREENING

50 µL of subcultures (see section 2.2.2.3) was added into the wells of black 96-well plates (Corning Incorporated). 100 µL of PLB-985 suspension (3 x 10^5 cell/mL in clear RPMI-1640) was added to the appropriate wells of these black 96-well plates. 100 µL of clear RPMI-1640 was added to the wells without PLB-985. The plate was then incubated at 37°C and 5% CO_2 for 2.5 hours. After incubation, the alamarBlue™ assay was carried out (see section 2.2.5.5).

2.2.5.4 VALIDATION SCREEN OF SUSCEPTIBLE MUTANTS

50 µL of WT or mutant cell suspension [1.5 x 10^5 cells or MOI of 5, in clear RPMI-1640 with 1X (v/v) P/S/Q, 6% human pooled plasma and 0.15 µg/mL of doxycycline] was added to the wells of a black 96-well plate (Corning Incorporated). 100 µL of neutrophil suspension (3 x 10^5 cell/mL in clear RPMI-1640) was added to the wells of the black 96-well plate. 100 µL of RPMI-1640 was added to the wells without neutrophils. The plates were then incubated at 37°C and 5% CO_2 for 2.5 hours. After incubation, the alamarBlue™ assays were carried out (see section 2.2.5.5).

2.2.5.5 ALAMARBLUE™ ASSAY

After 2.5 hours of incubation, plates were centrifuged at 1000 x g for 3 minutes. 100 µL of supernatant was removed, and 50 µL of 0.1% Triton X-100 in ddH_2O was added to the wells. The plates were centrifuged at 1000 x g for 3 minutes after 5 minutes incubation with Triton X-100. 100 µL of supernatant was removed from the wells, and wells were washed three times with 100 µL of 1X (w/v) PBS. Lastly, 90 µL of 1X (w/v) PBS [with 1X (v/v) P/S/Q] and 10 µL of 10X (w/v) alamarBlue™ were added to each well, and then the plates were incubated at 37°C and 5% CO_2 for 14-15 hours. The FLUOstar Omega Microplate Reader (BMG LABTECH) was used to read the plate by recording fluorescence at 544/590 (gains: 1000). Then the percentage (%) of metabolic rate was calculated based on alamarBlue™ fluorescence signals:

$$\% = \left(\frac{(C.albicans + \text{Neutrophils or PLB\_985}) - (\text{Neutrophils or PLB\_985 only})}{(C.albicans \text{ only}) - (\text{RPMI\_1640 only})} \right) \text{x } 100$$

2.2.5.6 CALCOFLUOR WHITE ASSAY

The protocol is similar to the alamarBlue™ assay. However, 90 µL of 1X (w/v) PBS [with 1X (v/v) P/S/Q] and 10 µL of 10X (w/v) CFW were added to the well (after 14-15 hours of incubation) and incubated at room temperature for 10 minutes. Then, cells were fixed with 2% PFA for 20 minutes and washed two times with 100 µL of 1X (w/v) PBS. The FLUOstar Omega Microplate Reader (BMG LABTECH) was used to read the plate by recording fluorescence at 360/460 (gains: 500).

2.2.6 *C. ALBICANS* SERUM-INDUCED FILAMENTATION ASSAY

2.2.6.1 *C. ALBICANS* FOETAL BOVINE SERUM (FBS)-INDUCED FILAMENTATION ASSAY

C. albicans overnight cultures were diluted to an OD_{600} of 0.2 in YPD media (with 0.05 µg/mL of doxycycline). The diluted cultures were grown for a further 4 hours at 30 °C and 200 rpm. After 4 hours of incubation, the cultures were diluted to an OD_{600} of 0.2 in YPD media (with 0.05 µg/mL of doxycycline and 10% FBS) and incubated for 2 hours at 37 °C and 200 rpm. Leica DMI6000 wide-field microscopy [in differential interference contrast (DIC) mode] was used to take images of each culture before and after FBS was added. Then, the average morphology index (of 30 cells per image) was calculated (Merson-Davies and Odds, 1989).

2.2.7 *C. ALBICANS* OXIDATIVE STRESS ASSAY

2.2.7.1 *C. ALBICANS* H_2O_2 OXIDATIVE STRESS ASSAY

After 4 hours of incubation (see section 2.2.6), each *C. albicans* strain was diluted with 1X (w/v) PBS (with 0.05 µg/mL of doxycycline) to 2 x 10^4 cell/mL. Each cell solution was further divided into three 5 mL in glass culture tubes. Tube 1 was diluted 1: 10 in 1X (w/v) PBS (with 0.05 µg/mL of doxycycline), and 100 µL of the solution were plated onto three YPD agar plates. Tube 2 and 3 were incubated with or without 0.1 mM (w/v) H_2O_2, respectively, for 1 hour at 37 °C and 200 rpm. After incubation, each tube was diluted 1: 10 in 1X (w/v) PBS (with 0.05 µg/mL of doxycycline) and 100 µL of the solution were plated onto three YPD agar plates. YPD agar plates were incubated at 30 °C for 2 days, and the survival rate of each strain was calculated based on the colony-forming unit (CFU) counts.

2.2.8 *C. ALBICANS* CELL WALL STRESS ASSAY

2.2.8.1 *C. ALBICANS* CELL WALL STRESS ASSAY USING CALCOFLUOR WHITE AND CONGO RED

After 4 hours of incubation (see section 2.2.6), *C. albicans* was diluted with 1X (w/v) PBS (with 0.05 µg/mL of doxycycline) to an OD_{600} of 0.1. It was then further diluted to six serial 10-fold dilutions in 1X (w/v) PBS (with 0.05 µg/mL of doxycycline). 5 µL of each dilution was spotted onto the following agar plates (pH 5.5 – 7.0): YPD control, 75 µg/mL of Congo Red YPD, 100 µg/mL of CFW YPD. These plates were incubated at 30 °C for 3 days.

2.2.9 OXIDATIVE BURST ASSAY

2.2.9.1 MEASURE OXIDATIVE BURST USING LUMINOL METHOD

$1x10^5$ PLB-985 or neutrophils were seeded in 100 µL of ROS media [HBSS (with Ca^{2+} and Mg^{2+}), 1X (v/v) HEPES and 0.025% (v/v) HSA] in a white 96-well plate (Costar). 11 µl of a mixture of HRP (stock concentration of 1200 U/mL) and Luminol (stock concentration of 50 mM) diluted 1:200 in ROS media was added to the cells, and the plate was incubated at 37 °C and 5% CO_2 for 15 minutes. After that, cells were stimulated with either 100 nM of Phorbol 12-myristate 13-acetate (PMA) or *C. albicans* (MOI of 5) or unstimulated. The FLUOstar Omega Microplate Reader (BMG LABTECH) was used to read the plate by recording chemiluminescence for 4 hours in 2 minutes intervals. The area under the ROS kinetic curves was calculated to determine total ROS production.

2.2.10 NETOSIS ASSAY

2.2.10.1 MEASUREMENT OF NEUTROPHIL EXTRACELLULAR TRAP (NET) FORMATION USING SYTOX™ DYES

100 µL of WT or mutant cell suspension [MOI of 5, in NETs assay media (0.025% HSA, 10mM HEPES in clear RPMI-1640), with 2% human pooled plasma and 0.05 µg/mL of doxycycline] was added to the wells of a black 96-well plate (Corning Incorporated) and incubated at 37°C and 5% CO_2 for 2 hours (until exhibit filamentous morphology). Then, 50 µL of neutrophil suspension (6 x 10^5 cell/mL in NETs assay media, with 2% human pooled plasma and 0.05 µg/mL of doxycycline) was added to the wells of the black 96-well plate. The plate was then incubated at 37°C and 5% CO_2 for 2 hours. After incubation, 1 µM of SYTOX™ Orange and 100 nM of SYTO™ Green were added to all wells, and the plate was imaged using EVOS FL Cell Imaging System (Thermo Fisher Scientific). Then, the number of NETs per image was calculated based on SYTOX™ Orange images.

2.2.11 STATISTICAL ANALYSIS

GraphPad Prism 9 was used to assess the Gaussian distribution of all data sets, using the Shapiro-Wilk test and Q-Q plot, and all data sets were found to be normally distributed. For group comparison, One- or Two-way ANOVA (depending on each data set condition) was used except for those with unequal variance where Welch's t-test was performed. Multiple t-test analysis was used to compare two groups over a time course. In all cases, $*p<0.05$ denoted statistical significance.

CHAPTER 3: KILLING ASSAY OPTIMISATION

3.1 INTRODUCTION

Different methods exist to quantify *Candida* killing by neutrophils; they include colorimetric-based assays as well as colony-forming units (CFU) based assays (Cihlar and Calderone, 2009). For my project, I have chosen to perform a chemiluminescence-based killing assay due to the high throughput nature of my experiment using either alamarBlue™ cell viability reagent or Calcofluor white staining.

alamarBlue™ reagent is a resazurin-based solution. Resazurin is a cell-permeable non-toxic compound. The reagent is irreversibly reduced from non-fluorescent blue resazurin to highly fluorescent red resorufin by the respiration of active cells (Rampersad, 2012, Chen et al., 2015). This means that its fluorescence indicates cell metabolic activity, which correlates to cell viability (i.e., the greater the fluorescence, the more viable cells) (Rampersad, 2012). Calcofluor White (CFW) is a fluorescent deep blue dye that can detect *C. albicans* by binding to cellulose and chitin components of the cell wall (Harrington and Hageage, 2003). Its fluorescence signal correlates to cell abundance (i.e., the greater the fluorescence, the greater the cell population). Therefore, these two reagents represent promising candidates for the GRACE™ library screen.

Due to the high quantity of neutrophils required to screen the GRACE™ library that consisted of 2,357 mutants (Merk & Co.) and a limited supply of primary human neutrophils, the PLB-985 cell line was chosen to use in the initial library screen. PLB-985 is an acute myeloid

leukaemia cell derived from the HL-60 cell line. It can be differentiated to neutrophil-like cells by stimulating the cells with DMF (see section 2.2.3), which have been shown to have similar morphology and exert similar functions to neutrophils against pathogens (Pedruzzi et al., 2002, Pivot-Pajot et al., 2010). Thus, the PLB-985 cell has the potential to be used in the initial GRACE™ library screen as an alternative cell to neutrophils.

This chapter aimed to optimise a *C. albicans* killing assay for the GRACE™ library screen, with the following objectives:

- Optimising high throughput chemiluminescence-based killing assays using alamarBlue™ and CFW reagents
- Establishing PLB-985 cells as a model for *C. albicans* killing assay
- Establishing a suitable MOI of *C. albicans* for the killing assay

3.2 RESULTS

3.2.1 ALAMARBLUE™ REQUIRES A LONGER INCUBATION PERIOD THAN CALCOFLUOR WHITE

Before incorporating alamarBlue™ and CFW into the killing assay, it was important to optimise a suitable incubation period for each reagent. Therefore, live and heat-killed WT (CaSS1) *C. albicans* cells were incubated with either alamarBlue™ over 24 hours or CFW over 30 minutes, according to the recommended times stated in the instruction manuals.

The alamarBlue™ fluorescence (RFU) signal of live cells continuously increased over the 24 hours of incubation, while this increase was not detected in heat-killed cells (Figure 3.1a). Live cells at a concentration of 1.5 x 10^6 cell/mL and 3 x 10^6 cell/mL appeared to have a significantly greater fluorescence (RFU) signal than the heat-killed cells at the same concentration from 12 hours and 8 hours onwards, respectively (Figure 3.1a.). Interestingly, this phenomenon was not observed in cells at a 3 x 10^5 cell/mL concentration.

The CFW fluorescence (RFU) signal of both live and heat-killed cells appeared to decrease continuously over 30 minutes of incubation (Figure 3.1b). This increase was not prominent in live cells compared to heat-killed cells as the fluorescent signal of all live cell concentrations had an overlap of the standard error of the mean (SEM) after 5 minutes. Unexpectedly, live cells at a concentration of 1.5 x 10^6 cell/mL and 3 x 10^6 cell/mL appeared to have a significantly greater fluorescence signal than the heat-killed cells at the same concentration after 10 and 5 minutes of incubation, respectively.

Therefore, I decided to choose 14-15 hours incubation time for alamarBlue™ and 10 minutes for CFW as part of my killing assay protocol.

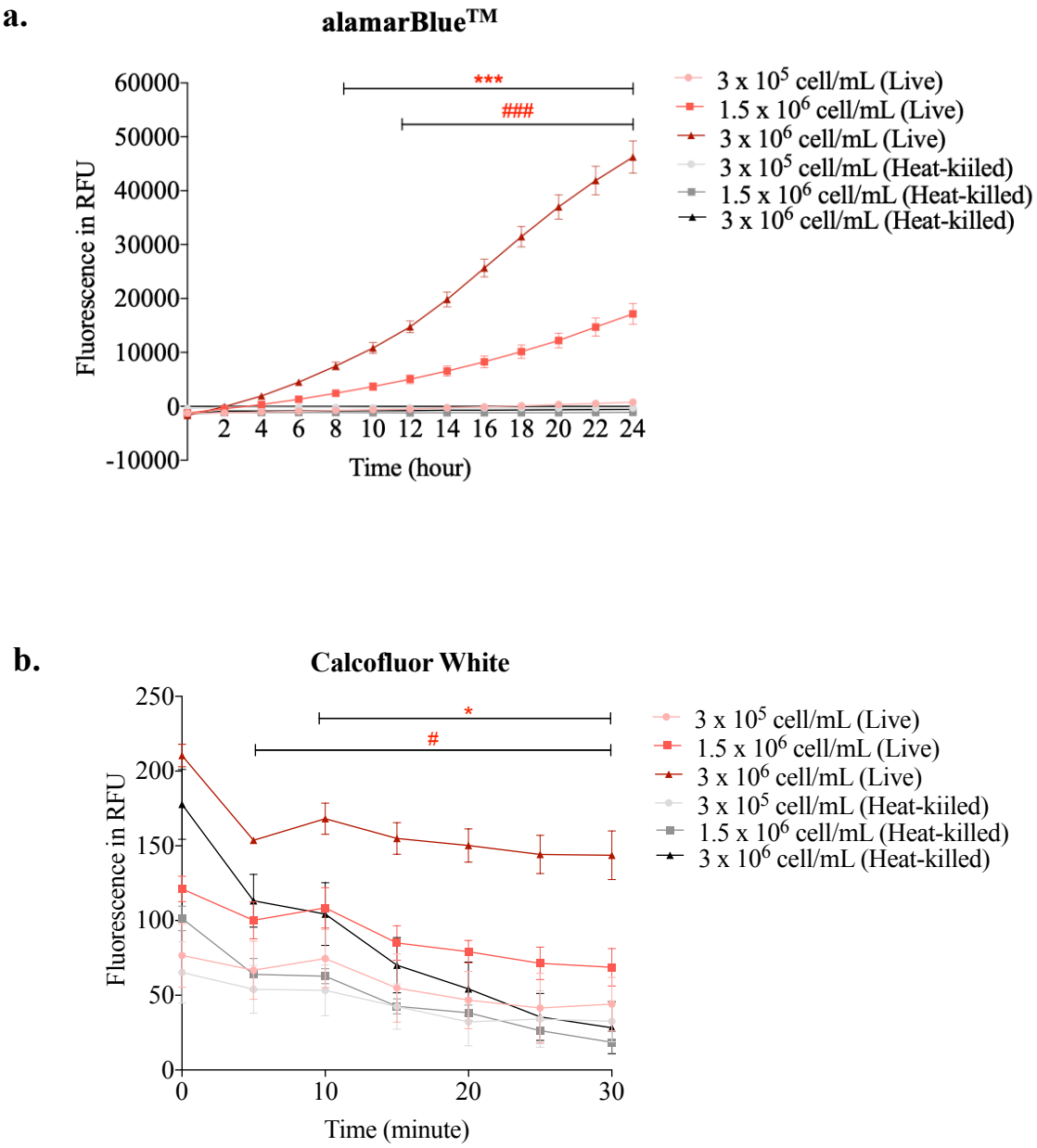


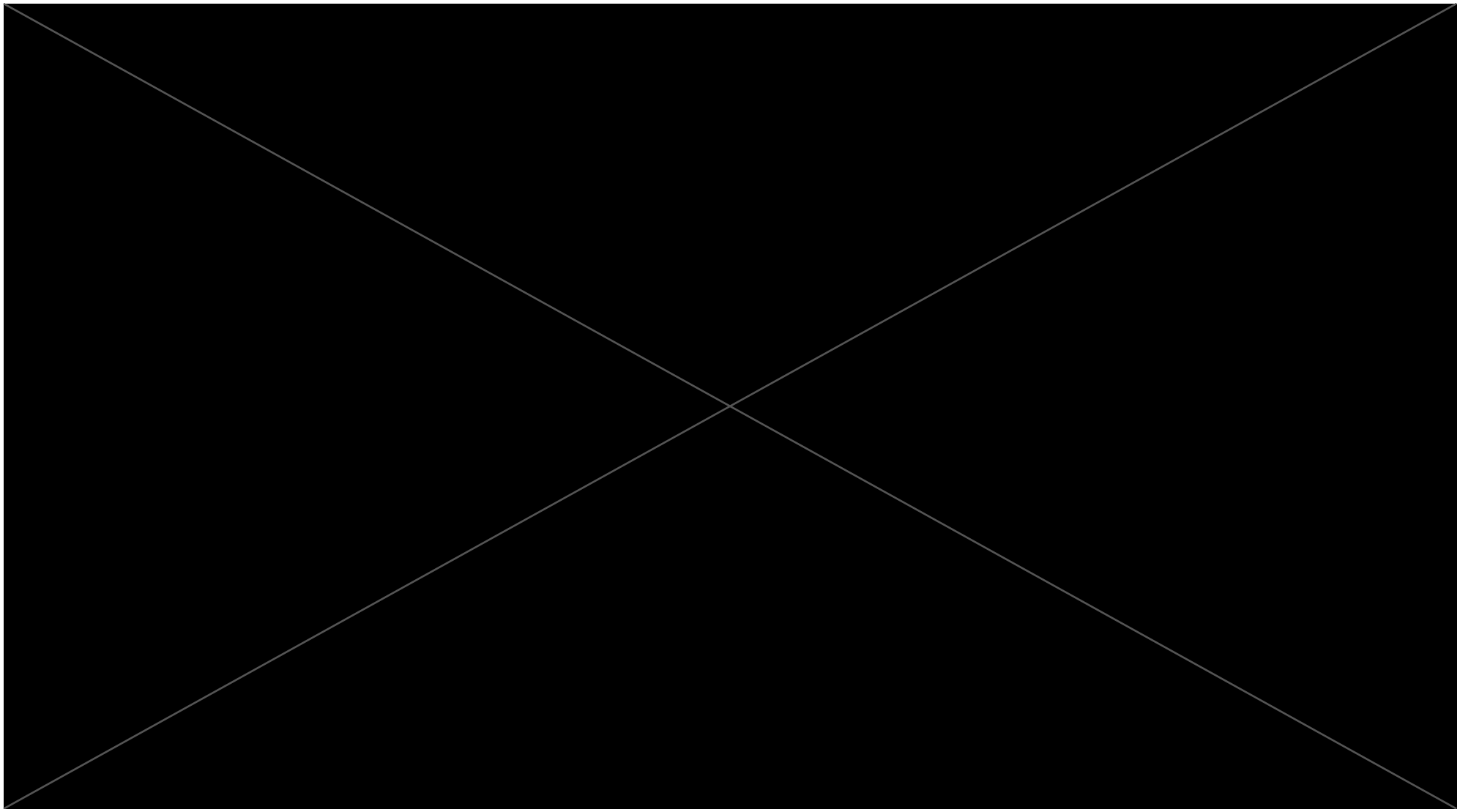
Figure 3.1: alamarBlue™ vs. Calcofluor White incubation periods. (**a.**) alamarBlue™ fluorescence of live and heat-killed WT (CaSS1) *C. albicans* over 24 hours. (**b.**) CFW fluorescence of live and heat-killed WT over 30 minutes. Fluorescence (mean in RFU) ± SEM (Standard error of mean). Statistical significance was calculated by 2-tailed multiple paired t-test comparison; #, * $p<0.05$, ###, *** $p<0.001$; * comparison between live and heat-killed 3 x 10^6 cell/mL; # comparison between live and heat-killed 1.5 x 10^6 cell/mL; $n=3$.

3.2.2 ALAMARBLUE™ REAGENT IS MORE SENSITIVE THAN CALCOFLUOR WHITE STAINING

Having established the incubation time for each reagent, I next examined their sensitivity in detecting *C. albicans* killing by PLB-985 and neutrophils. WT cells at the MOI of 1, 5 or 10 were co-incubated with or without either neutrophils or PLB-985, then either CFW or alamarBlue™ assays were carried out (see 2.2.5.2 and Figure 3.2).

The alamarBlue™ fluorescence signal of cells at the MOI of 5 treated with PLB-985 was significantly lower than those without PLB-985, while this difference was not detected for cells at the MOI of 1 and 10 groups (Figure 3.3a). Similarly, the alamarBlue™ fluorescence signals of cells at the MOI of 1 and 5 co-incubated with neutrophils were significantly lower than those without neutrophils (Figure 3.3b). However, there was no significant between the CFW fluorescence signals of cells at the MOI of 1, 5, 10 co-incubated with PLB-985 or neutrophils and without PLB-985 or neutrophils (Figure 3.3c-d).

Therefore, I decided to only use alamarBlue™ reagent as part of my GRACE™ library screen as the data revealed that alamarBlue™ has a higher sensitivity at detecting neutrophil killing of *C. albicans* compared to CFW.



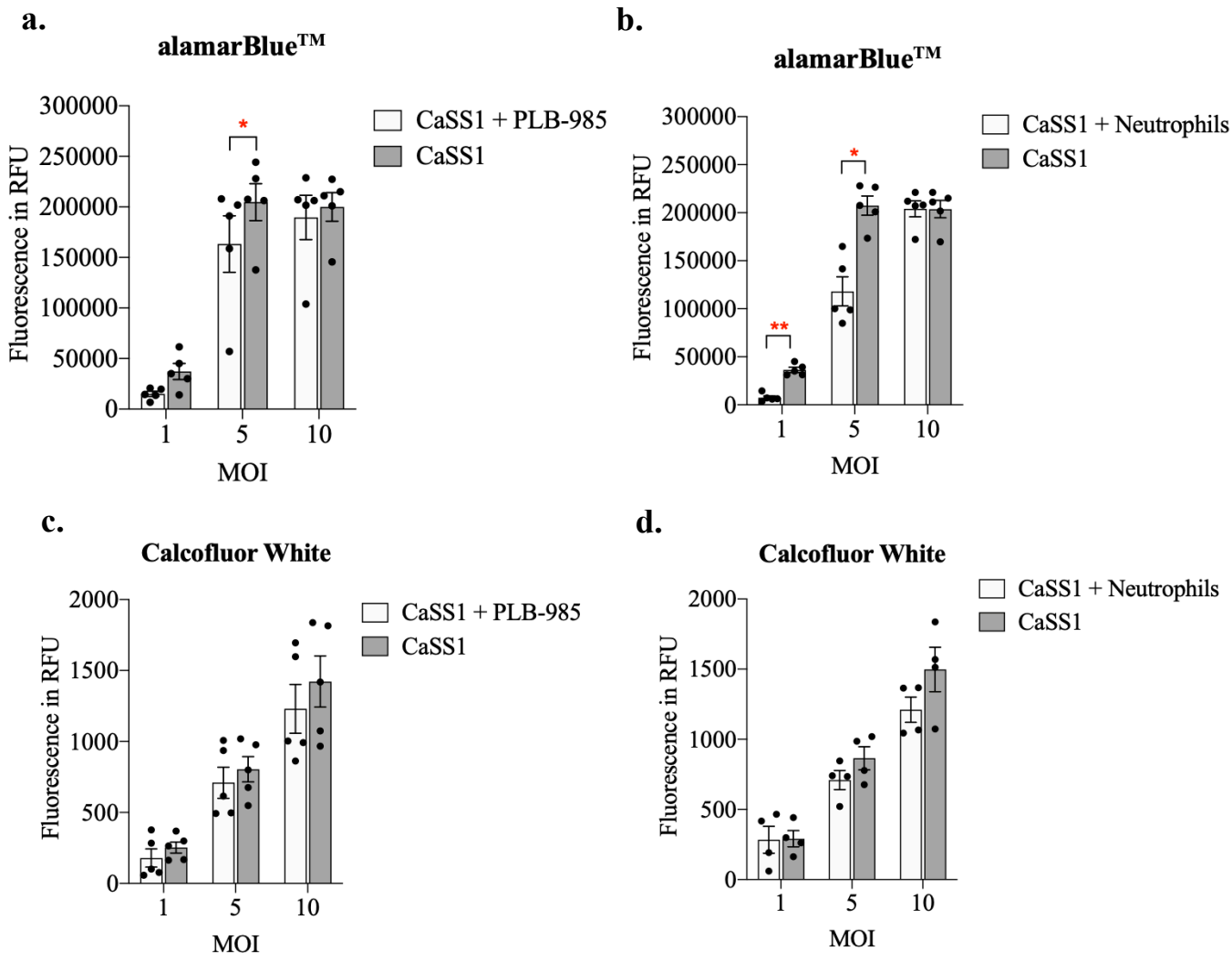


Figure 3.3: alamarBlue™ provided more sensitive detection of *C. albicans* killing by neutrophils than Calcofluor White (CFW). (**a**.) alamarBlue™ fluorescence of CaSS1-PLB-985 killing assay (KA). (**b.**) alamarBlue™ fluorescence of CaSS1-neutrophil KA. (**c**.) CFW fluorescence of CaSS1-PLB-985 KA. (**d.**) CFW fluorescence of CaSS1-neutrophil KA. Fluorescence (mean in RFU) ± SEM. Statistical significance was calculated by 2-way ANOVA; * $p<0.05$, ** $p<0.01$; $n=5$ (except for CFW CaSS1-neutrophil KA where $n=4$).

3.2.3 PLB-985 CELLS ARE SIMILAR TO PRIMARY HUMAN NEUTROPHILS AT KILLING *C. ALBICANS*

To establish PLB-985 effectiveness at killing *C. albicans* compared to neutrophils, WT *C. albicans* cells were co-incubated with or without either PLB-985 or neutrophils. After the killing assay with alamarBlue™ was carried out (see section 2.2.5), the percentage metabolic rate of *C. albicans* was calculated based on the fluorescence signals of PLB-985/neutrophil and non-PLB-985/neutrophil treatment groups.

The data revealed that the higher the MOI, the greater the percentage metabolic rate of WT strain (Figure 3.4). This, in turn, suggested that there were more viable *C. albicans* cells at a higher MOI after being challenged with neutrophils or PLB-985. WT cells that were challenged with neutrophils had a lower percentage metabolic rate at the MOI of 1 (by ~23%) and 5 (by ~20%), and a higher percentage metabolic rate at the MOI of 10 (by 7%) than those that were challenged with PLB-985. However, these differences were not statistically significant except for the MOI of 1 (Figure 3.4). Therefore, this suggested that PLB-985 cells are only slightly less efficient at killing *C. albicans* as neutrophils.

Having shown that PLB-985 cells were similar to neutrophils at killing *C. albicans,* I next investigated a suitable *C. albicans* cell ratio to neutrophils/PLB-985 for the GRACE™ library screen. The MOI with the closest to 50% metabolic rate was chosen to use in my screen, as this would allow a change in either direction to be detected to determine whether a mutant was susceptible to PLB-985 or neutrophils compared to WT strain. MOI of 5 was chosen for both PLB-985 and neutrophils.

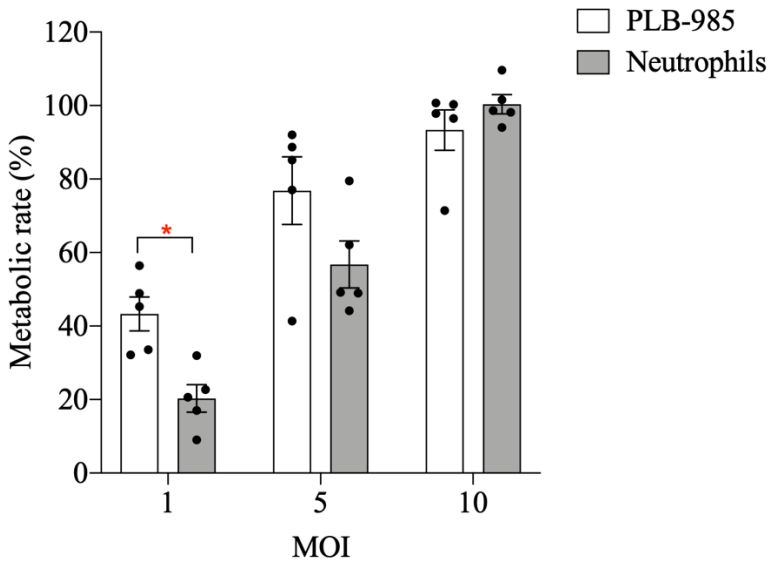


Figure 3.4: PLB-985 exhibited a similar level of C. *albicans* killing to neutrophils. The graph shows the metabolic rate (%) of CaSS1 with either PLB-985 or neutrophils at MOI of 1, 5 and 10. % (mean) ± SEM. Statistical significance was calculated by 2-way ANOVA; *$p<0.05$; $n=5$.

3.2.4 PLB-985 CELLS ARE SIMILAR TO NEUTROPHILS AT PRODUCING REACTIVE OXYGEN SPECIES

Having established a suitable MOI, respiratory burst assays were carried out to assess another function of PLB-985 compared to neutrophils by challenging PLB-985 or neutrophils with *C. albicans* (WT) at the MOI of 5. Reactive oxygen species (ROS) production is one of the main neutrophil antifungal responses against *C. albicans* (Jamieson et al., 1996, Segal, 2005). Therefore, it was important to examine the ability of PLB-985 cells to produce ROS.

The kinetic curves showed that PLB-985 produced an earlier ROS burst with an overall lower ROS chemiluminescence signal (~ 1/3 less based on ROS peak values) than neutrophils when stimulated with PMA, a potent non-physiological chemical inducer (Figure 3.5a). Similarly, when stimulated with *C. albicans*, PLB-985 started to produce a ROS burst simultaneously with neutrophils but with an overall lower ROS chemiluminescence signal (~ 1/2 less based on ROS peak values) (Figure 3.5c). These effects were also observed when areas under the kinetic curve were calculated. However, the area under the kinetic curve showed no significant difference in the total ROS production between PLB-985 and neutrophils when stimulated with PMA or *C. albicans* (Figure 3.5b, d). This implied that PLB-985 cells were able to produce ROS, but not as well as neutrophils. This may also explain their reduced ability to kill *C. albicans*.

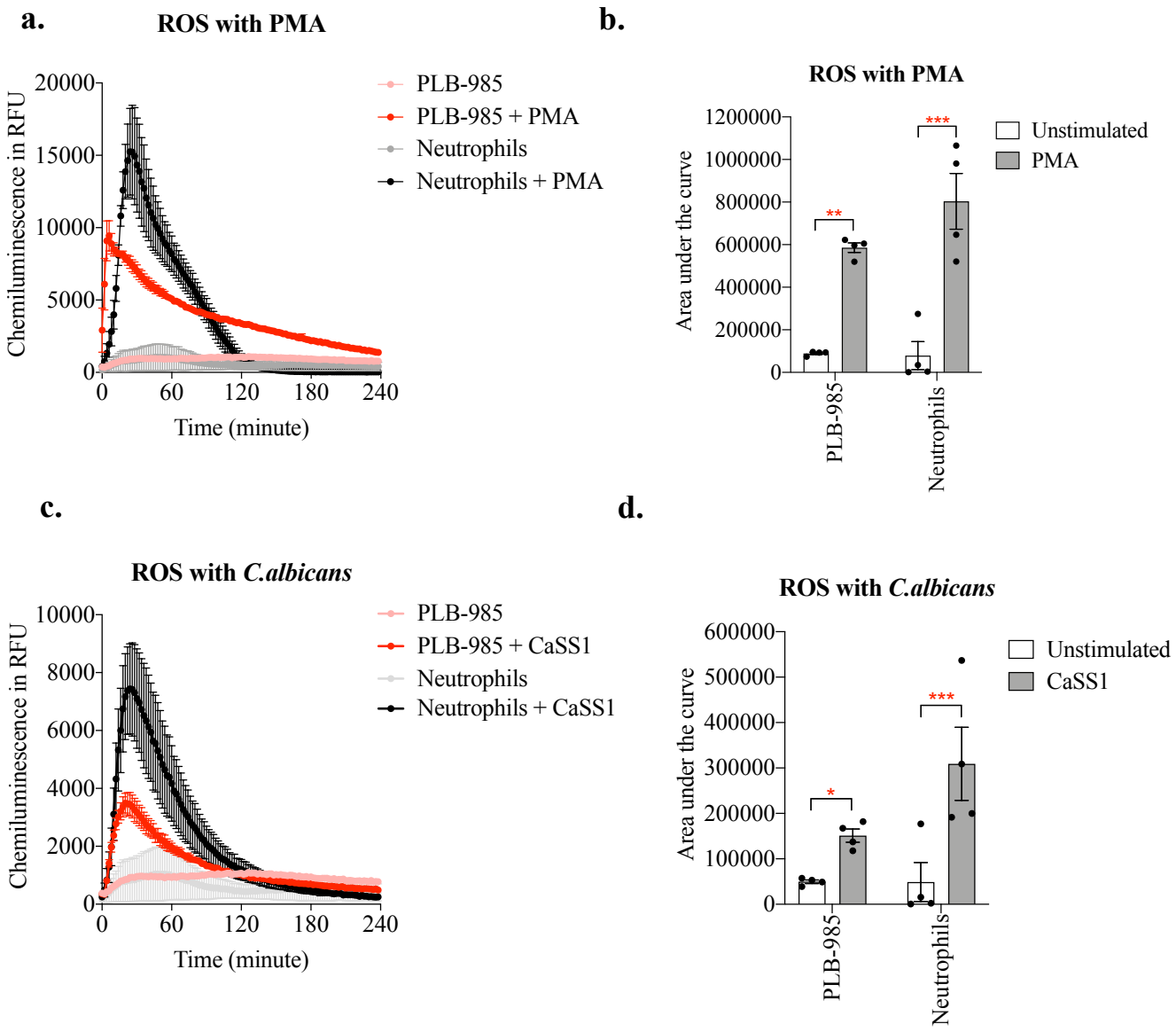


Figure 3.5: PLB-985 cells showed reduced respiratory burst against *C. albicans* compared to neutrophils. (**a.**) Chemiluminescence of reactive oxygen species (ROS) signal of PLB-985 or neutrophils with PMA. (**b.**) The area under the ROS curve of (a). (**c.**) Chemiluminescence of ROS signal of PLB-985 or neutrophils with CaSS1. (**d.**) The area under the ROS curve of (c). Chemiluminescence (mean in RFU) ± SEM; Area under the curve (mean) ± SEM. Statistical significance was calculated by 2-way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$; $n=4$. PLB-985 and neutrophils were challenged with WT *C. albicans* at MOI of 5 (see section 2.2.9).

3.3 DISCUSSION

The studies presented in this chapter aimed to optimise the *C. albicans* killing assay for the GRACE™ library screen and establish PLB-985 as a potential model in the *C. albicans* killing assay.

alamarBlue™ fluorescence signal correlates to cell metabolic activity, indicating cell viability as only active cells can reduce the alamarBlue™ from non-fluorescent blue to fluorescent red colour (Rampersad, 2012, Chen et al., 2015). The CFW fluorescence signal correlates to biomass abundance (of live or dead cells) as it only stains the fungal cell wall (Harrington and Hageage, 2003). I initially anticipated that if more *C. albicans* cells survived after being challenged with PLB-985 and neutrophils, there would be more cell wall surface to bind to the CFW. Thus, cell abundance based on CFW staining may be able to predict cell viability.

14-15 hours of alamarBlue™ incubation was chosen as my data revealed that this incubation period was sufficient to distinguish between live and heat-killed cells. However, later incubation time points offered a greater significant difference, this time point was chosen for the killing assay to prevent the overshoot of "over incubation" of cells with alamarBlue™. My data revealed that the alamarBlue™ signal plateaued and decreased at a higher cell concentration over a more extended incubation period (Supplementary figure 1a). It is not well understood why this phenomenon occurred as the reduction of resazurin to resorufin is irreversible (Chen et al., 2015). However, a plateau signal was expected as all resazurin in the solution has been reduced. One plausible explanation could be that over time *C. albicans* started to change their metabolism and switch on anaerobic respiration due to cell stress in response to overpopulation and lack of nutrients. This, in turn, may have caused the media to

become acidic and cause resorufin to reversibly reduce to the non-fluorescent dihydroresorufin (çakır and Arslan, 2010, Chen et al., 2015). Another explanation could be that *C. albicans* may have the ability to break down resazurin. Due to the extensive screening scale of the GRACE™ library (96 mutants per plate), it was impossible to dilute each mutant to the exact required MOI number. This means that more or fewer cells might be added to the wells, and thus would affect the alamarBlue™ reduction reaction (see section 2.2.2.2 and Chapter 4). Also, the metabolic activity of each mutant in the GRACE™ library was unknown. This is another important factor that could affect the reduction rate of resazurin. Therefore, 14-15 hours of alamarBlue™ incubation was chosen as it provided good resolution and avoided fungal overgrowth.

10 minutes of CFW incubation was chosen as my data revealed no or little difference between fluorescence signals after 5 minutes for live cells. Thus, this suggested that this incubation period was sufficient for the dye to stain all cell walls. However, all samples showed decreased fluorescence over time. This could be due to over and frequent excitation, which causes CFW dye to lose its fluorescence signal. However, this does not fully explain the dramatic decrease of the heat-killed cells. Interestingly, live cells at a higher cell concentration had a significantly higher fluorescence signal than heat-killed cells (Supplementary figure 1b). This was an unexpected observation as *C. albicans* cells were only incubated for a short period and not at their optimum growth conditions (see section 2.2.5.1). Therefore, it was unlikely for the live cells to outgrow the heat-killed cells in the given 30 minutes. One explanation for this observation could be that high temperature perhaps caused *C. albicans* to lose its ability to maintain cell wall integrity and caused cell wall structure alterations, for example, by inducing the breakdown of *C. albicans* cell wall components (such as β-glycans and glycoproteins) or denaturing enzymes that are required to maintain cell wall structure (Garcia-Rubio et al., 2019,

Gazendam et al., 2016). Therefore, the dye may have been unable to bind to the cell wall properly and hence a lower signal.

The alamarBlue™ killing assay with WT cells revealed significant killing of *C. albicans* at the MOI of 5 by PLB-985. However, the CFW killing assay with WT cells showed no killing of *C. albicans* by PLB-985. Since both reagents did not give a similar result in terms of reporting PLB-985 ability to kill *C. albicans*, the question of each reagent sensitivity was raised. It was impossible to compare reagent sensitivity based on PLB-985 killing assay data as it was unknown if PLB-985 cells can effectively kill *C. albicans*. Therefore, *C. albicans* killing assays with primary human neutrophils were performed to compare the dyes' sensitivity. Neutrophils are cells that have been confirmed to have the ability to kill *C. albicans* (Gazendam et al., 2016). The alamarBlue™ killing assay reported a significant killing of *C. albicans* at the MOI of 1 and 5, while this observation was not reported in the CFW killing assay. This showed that the alamarBlue™ reagent was more sensitive than CFW at detecting *C. albicans* killing by PLB-985 and neutrophils. This observation was expected because the alamarBlue™ fluorescence signal directly correlates to cell viability. Although CFW has a higher specificity to *C. albicans*, it failed to detect cell viability. One possible explanation for this outcome could be that the neutrophils failed to degrade *C. albicans* components of dead cells completely. A study showed that neutrophils could release NETs to trap and kill *C. albicans,* as an alternative killing method to phagocytosis, a "traditional" mechanism that degrades *C. albicans* components (Branzk et al., 2014). This means there will be dead *C. albicans* cells with intact cell walls, which will get picked up by the CFW fluorescence signal. Thus, alamarBlue™ was chosen for the screen to detect *C. albicans* viability. However, it should be used with caution due to its low specificity.

To assess PLB-985 ability to kill *C. albicans*, cell viability was determined based on the percentage metabolic rates of *C. albicans* after being exposed to PLB-985 and neutrophils. The data revealed that PLB-985 cells were slightly less efficient at killing *C. albicans* than neutrophils at the MOI of 1 and 5 with ~23% and ~20% more viable cells, respectively. However, the difference was only statistically significant at the MOI of 1. The data also showed no or negligible killing at the MOI of 10 for both PLB-985 and neutrophils. This is likely because there were too many *C. albicans* cells to be killed. Also, these *C. albicans* cells will exert "anti-neutrophil" responses to escape from neutrophil killing (see section 1.3). Thus, it will be more challenging for neutrophils or PLB-985 to deal with a great abundance of *C. albicans*. Overall, the killing assay data revealed that PLB-985 could effectively kill *C. albicans* depending on the MOI of *C. albicans.*

MOI of 5 was chosen for both PLB-985 and neutrophils as the most appropriate for the GRACE^{TM} library screen. It offered the closest to 50% metabolic rate for neutrophils, allowing changes in either direction to be detected. For PLB-985 cells, the metabolic rate was ~77% at the MOI of 5. According to the percentage metabolic rate, the MOI of 5 is not an ideal choice for PLB-985 compared to the MOI of 1 that had ~57% metabolic rate. However, there was no significant *C. albicans* killing by PLB-985 cells at the MOI of 1, which suggested that the MOI of 1 was not a suitable MOI despite having a value closer to 50%.

Previous research demonstrated that PLB-985 cells could perform similar functions to neutrophils, such as the ability to perform phagocytosis, ROS production and NETosis to some pathogens and stimuli (Pedruzzi et al., 2002, Pivot-Pajot et al., 2010, Volk et al., 2011). The ROS kinetic curves showed that PLB-985 cells could produce a ROS burst in response to *C. albicans* and PMA stimulation with an overall lower total ROS production and ROS peak than

neutrophils. Interestingly, the area under the kinetic curves revealed that the difference in ROS production between neutrophils and PLB-985 cells was not statistically significant. It is worth mentioning that the area under the curve is correlated to the total ROS production. Therefore, it does not give any information on how potent or toxic the ROS contents produced by PLB-985 are to *C. albicans* compared to neutrophils. The concentration of ROS in a specific time point (e.g., the peak of the kinetic curve) may give a better indication of the ROS potency and account for the reduced killing observed in PLB-985.

3.4 CONCLUSION

My data revealed that PLB-985 cells were a good model to use in the initial GRACE^{TM} library screen as they were potent at killing *C. albicans*. As they are originated from a single clone and grown in the laboratory, there are fewer genetic variations and differences in *in vivo* priming between PLB-985 cells than neutrophils of different donors, which could influence how cells respond to pathogens.

This project is the first study to date to establish PLB-985 cells as a model for *C. albicans* killing assays and reveal their potential to be used in place of neutrophils when these are unavailable. Also, this is one of the first investigations to date (if not the first) to report the interaction between PLB-985 cells and *C. albicans*, such as their ability to kill *C. albicans* and produce ROS in response to *C. albicans*.

CHAPTER 4: THE GRACE™ LIBRARY SCREEN

4.1 INTRODUCTION

The GRACE™ (gene replacement and conditional expression) library is a non-redundant library that contains 2,357 *C. albicans* mutants (one mutant per gene) (Merk & Co). The library was created based on the tetracycline (Tet)-regulatable promoter system, whereby one allele of a target gene (of a diploid *C albicans* strain called CaSS1) was deleted, and the other copy was then placed under the control of a Tet repressive promoter. Tet analogues, such as doxycycline, inhibit target gene expression completely and thus inhibit its downstream functions (Roemer et al., 2003). Previous research successfully utilised the GRACE™ library to reveal novel information about *C. albicans* functions and its interaction with other immune cells, such as macrophages (Edouarzin et al., 2019, Lee et al., 2016, O'Meara et al., 2018, O'Meara et al., 2015, Xu et al., 2019). Therefore, screening the GRACE™ library with PLB-985 or neutrophils could help us better understand *C. albicans* interaction with neutrophils.

The research aim of this chapter was to identify *C. albicans* genetic factors that are important for the survival of neutrophil antifungal responses by screening the GRACE™ library. This chapter research objectives were:

- Estimate a suitable subcultured volume of GRACE™ library mutants to achieve an MOI of 5
- Identify GRACE™ library mutants that were susceptible to PLB-985 killing
- Validate the GRACE™ mutants that were susceptible to PLB-985 killing with neutrophils

4.2 RESULTS

4.2.1 THE OPTIMISATION OF A REQUIRED SUBCULTURED VOLUME FOR THE GRACE™ LIBRARY SCREEN

As the GRACE™ library is arrayed in a 96-well plate format (i.e., each well represents one mutant), it was not possible to dilute each strain to the MOI of 5 when performing the killing assay. To overcome this challenge, CaSS1 (WT) was incubated overnight and subcultured in a 96-well plate (see section 2.2.2.2). After subculturing, the OD_{595} of the plate was measured and used to calculate the number of cells, using OD_{595} of 1 = 1 x 10^7 cell/mL. The estimated volume of *C. albicans* suspension (containing MOI of 5 or 150,000 cells) was further determined. Figure 4.1 showed that the greater the dilutions, the greater the volume required to achieve an MOI of 5. For convenience purposes, 50 µL of undiluted subculture was chosen to add to the killing assay plate.

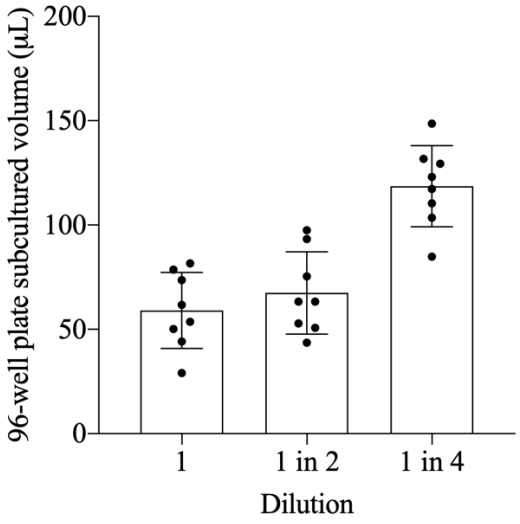


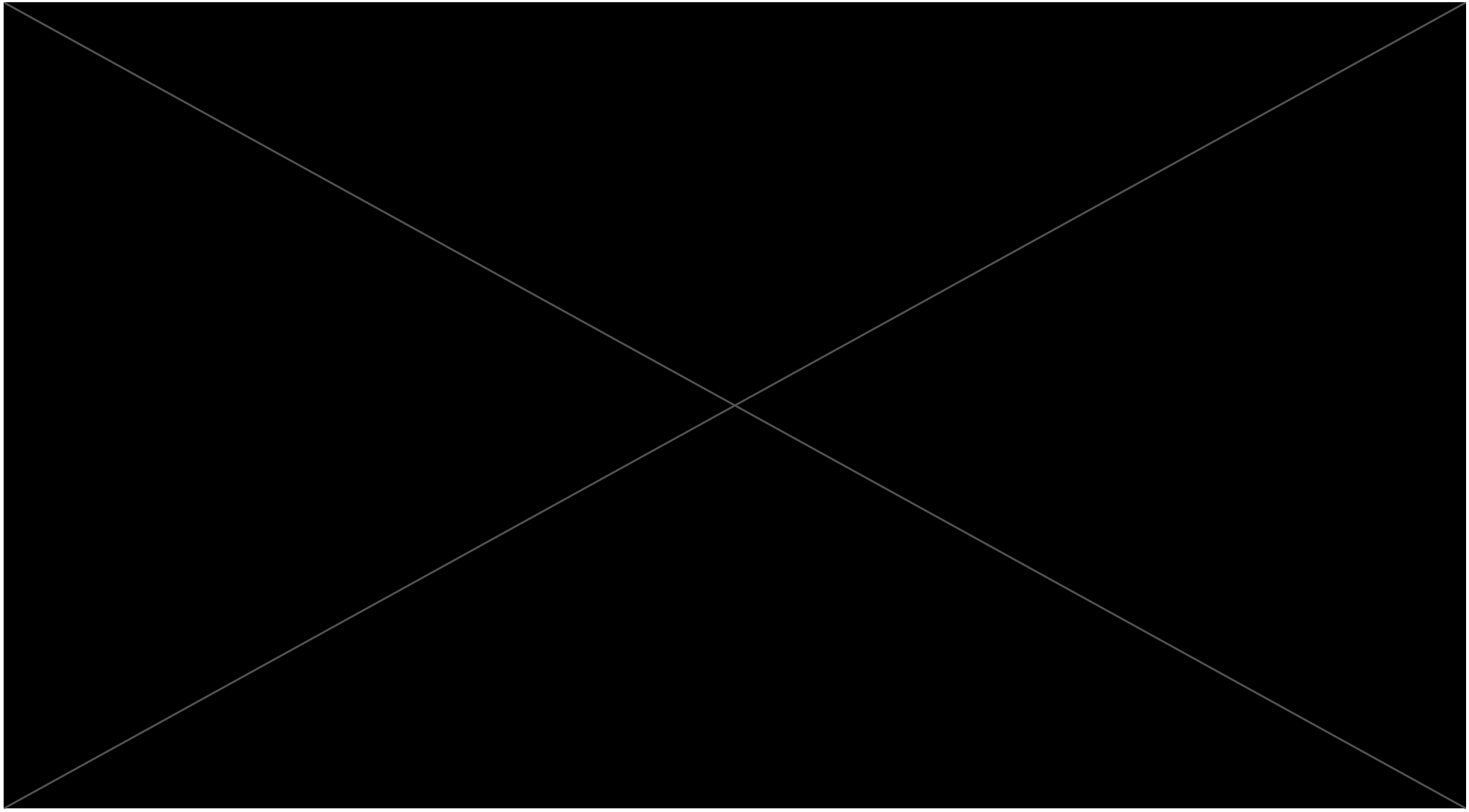
Figure 4.1: The required GRACE™ volume to achieve MOI of 5. The graph shows the estimated volume of *C. albicans* subcultured suspension containing 150,000 cells. Volume (mean in µL) ± Standard deviation (SD); *n=1*. Each dot represents a technical replicate.

4.2.2 THE GRACE™ LIBRARY MUTANTS ARE SUSCEPTIBLE TO PLB-985 KILLING

To identify *C. albicans* mutants susceptible to neutrophils, I first screened the GRACE™ library with PLB-985 cells and determined the mutant metabolic rate based on the alamarBlue™ fluorescence signal (see section 2.2.5.3 and Figure 4.2). The percentage metabolic rate is correlated to cell viability. Therefore, mutants susceptible to PLB-985 (or neutrophils) would have a lower percentage metabolic rate than the WT control.

Figure 4.3-4.6 show the percentage metabolic rate of *C. albicans* mutants from GRACE™ plates 1, 2, 7 and 11 with their internal WT controls (these plates were randomly selected). Six mutants (A12, B12, E07, G06 and G12) from plate 1 had a lower percentage metabolic rate than their internal WT control, while ~58 mutants had a higher percentage metabolic rate than the control (Figure 4.3). Similarly, 2 mutants (F12 and H11) from plate 2 had a lower percentage metabolic rate than their internal control, while ~46 mutants had a higher percentage metabolic rate than the control (Figure 4.4). Likewise, 7 mutants (A09, D07, F02, G03, H0, H04 and H11) from plate 7 were found to have a lower percentage metabolic rate than their WT control, while ~52 mutants had a higher percentage metabolic rate than the control (Figure 4.5). Comparably, 8 mutants (A01, A03, B01, B02, B05, B10, C01 and C02) from plate 11 were shown to have a lower percentage metabolic rate than their internal control, while ~42 mutants had a higher percentage metabolic rate than the WT (Figure 4.6). Interestingly, ~36% of all mutants screened across the four plates were found to have >100% metabolic rate (41 mutants from plate 1, 29 mutants from plate 2, 42 mutants from plate 7 and 28 mutants from plate 11).

The 23 *C. albicans* mutants (as summarised in Table 4.1) with greater susceptibility to PLB-985 cells were chosen to be further validated with neutrophils. As more than 50% of all mutants screened had a greater percentage metabolic rate than the WT control, I also randomly chose 2 mutants with a small standard error of the mean (D07 and G02 from plate 2) to be validated with neutrophils. Not only did these 2 mutants have a greater percentage metabolic rate than their internal WT control, but they also had an unusual >100% metabolic rate. Validating these two mutants could help me to assess the potential use of my optimised GRACE^{TM} screen assay as a method to identify resistant mutants to neutrophils.



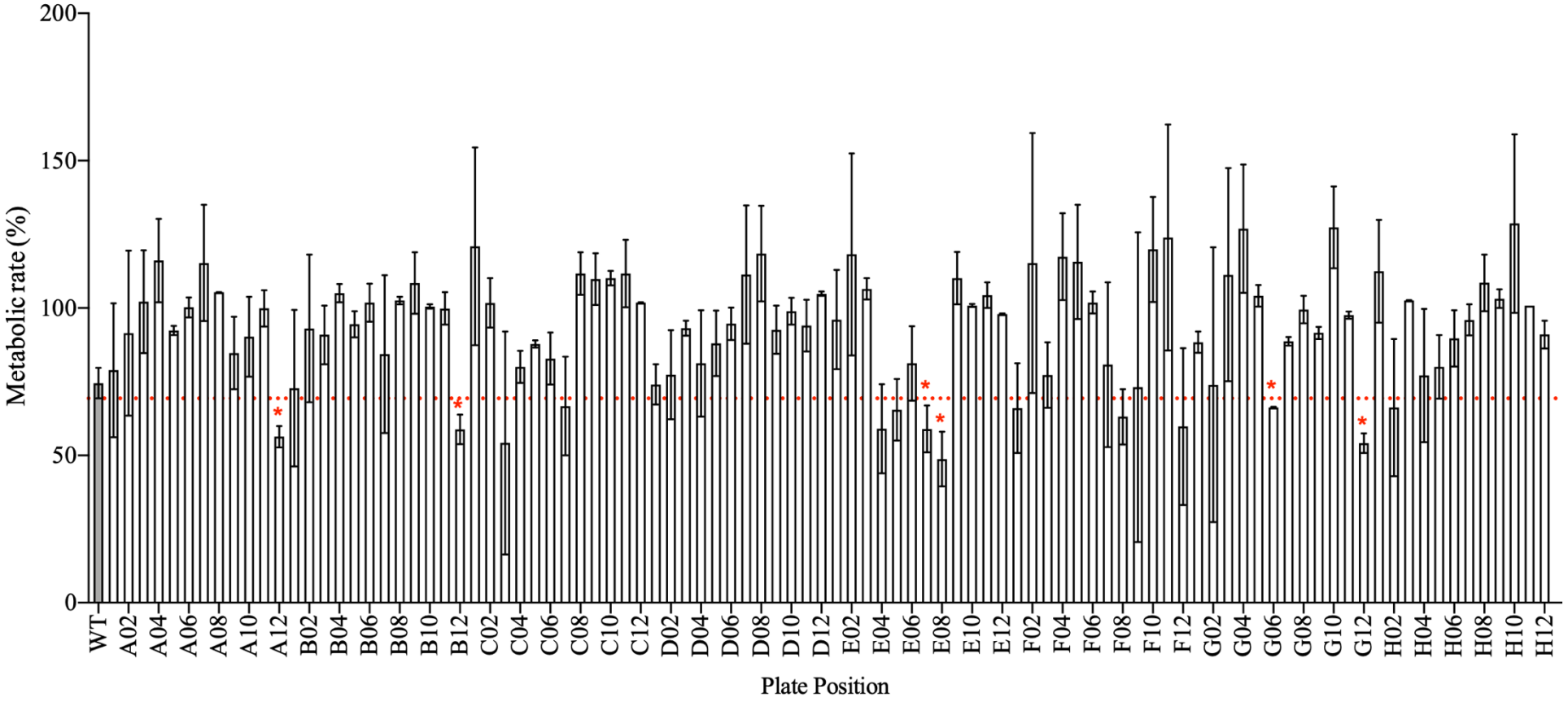


Figure 4.3: Percentage metabolic rate of GRACE^{TM} library plate 1 mutants post-exposure to PLB-985. The GRACE^{TM} killing assays with PLB-985 cells were performed, and the percentage (%) *C. albicans* metabolic rate was calculated based on alamarBlue^{TM} fluorescence signals (see section 2.2.5.3); % (mean) ± SEM; *n=2*. Mutants that were chosen to be further validated with neutrophils are denoted by '*'.

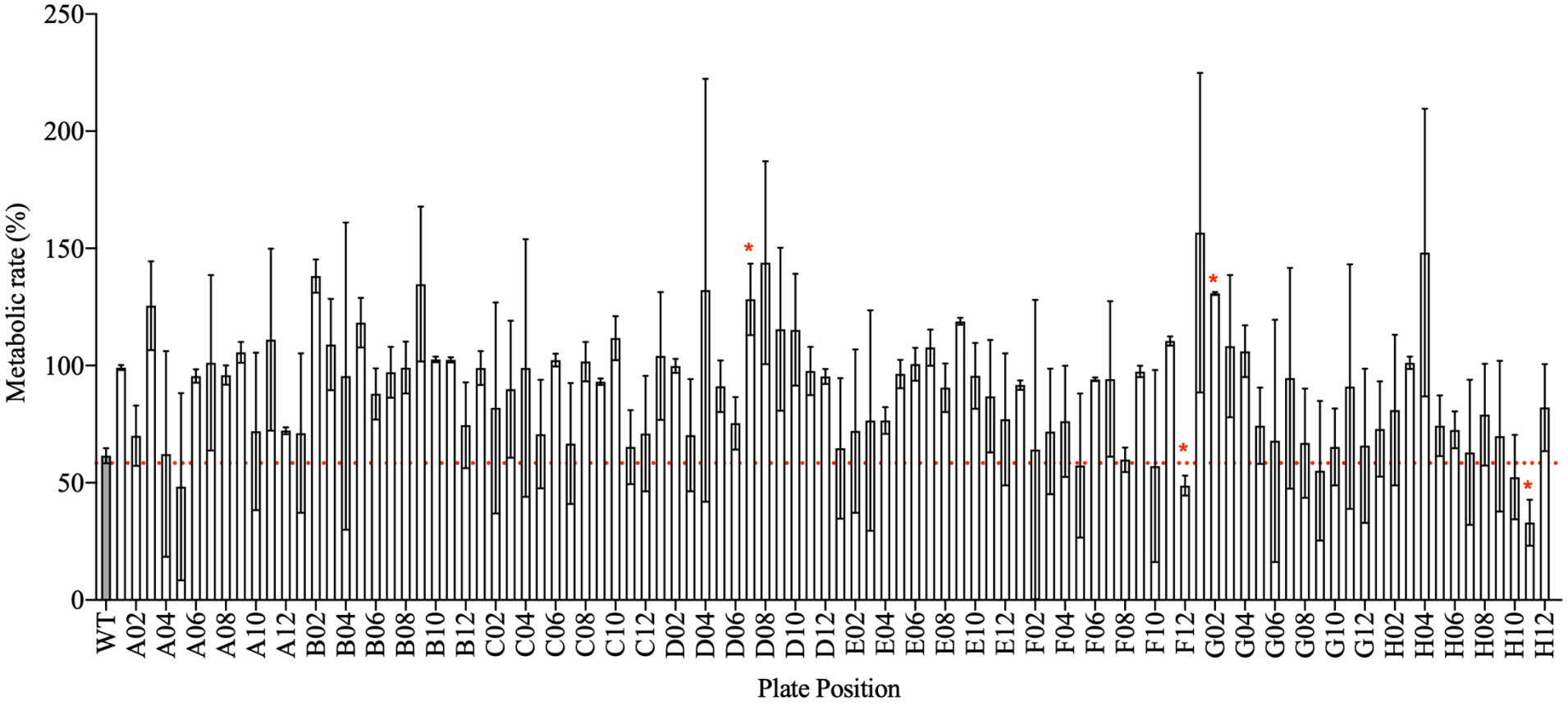


Figure 4.4: Percentage metabolic rate of GRACE^{TM} library plate 2 mutants post-exposure to PLB-985. The GRACE^{TM} killing assays with PLB-985 cells were performed, and the percentage (%) *C. albicans* metabolic rate was calculated based on alamarBlue^{TM} fluorescence signals (see section 2.2.5.3); % (mean) ± SEM; *n=2*. Mutants that were chosen to be further validated with neutrophils are denoted by '*'.

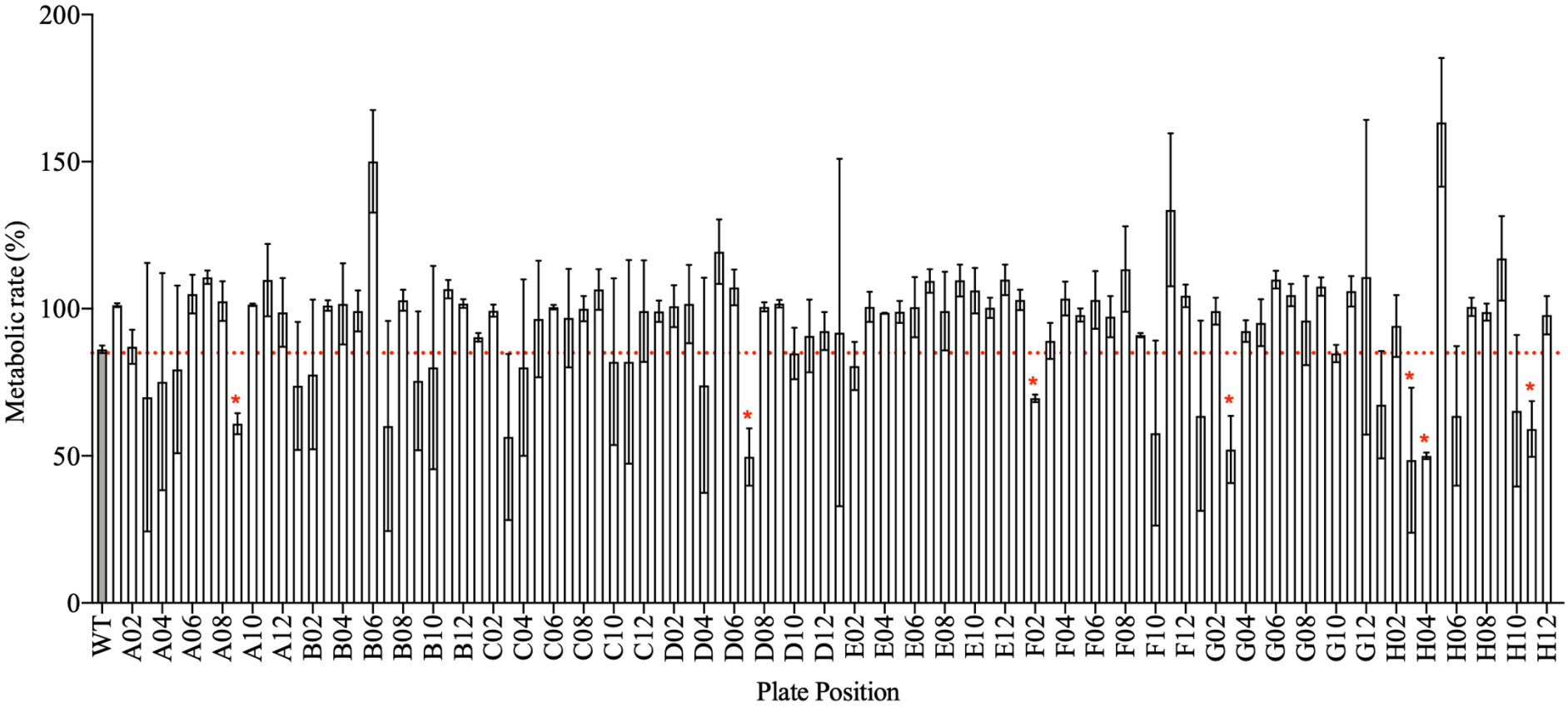


Figure 4.5: Percentage metabolic rate of GRACE™ library plate 7 mutants post-exposure to PLB-985. The GRACE™ killing assays with PLB-985 cells were performed, and the percentage (%) *C. albicans* metabolic rate was calculated based on alamarBlue™ fluorescence signals (see section 2.2.5.3); % (mean) ± SEM; *n=2*. Mutants that were chosen to be further validated with neutrophils are denoted by '*'.

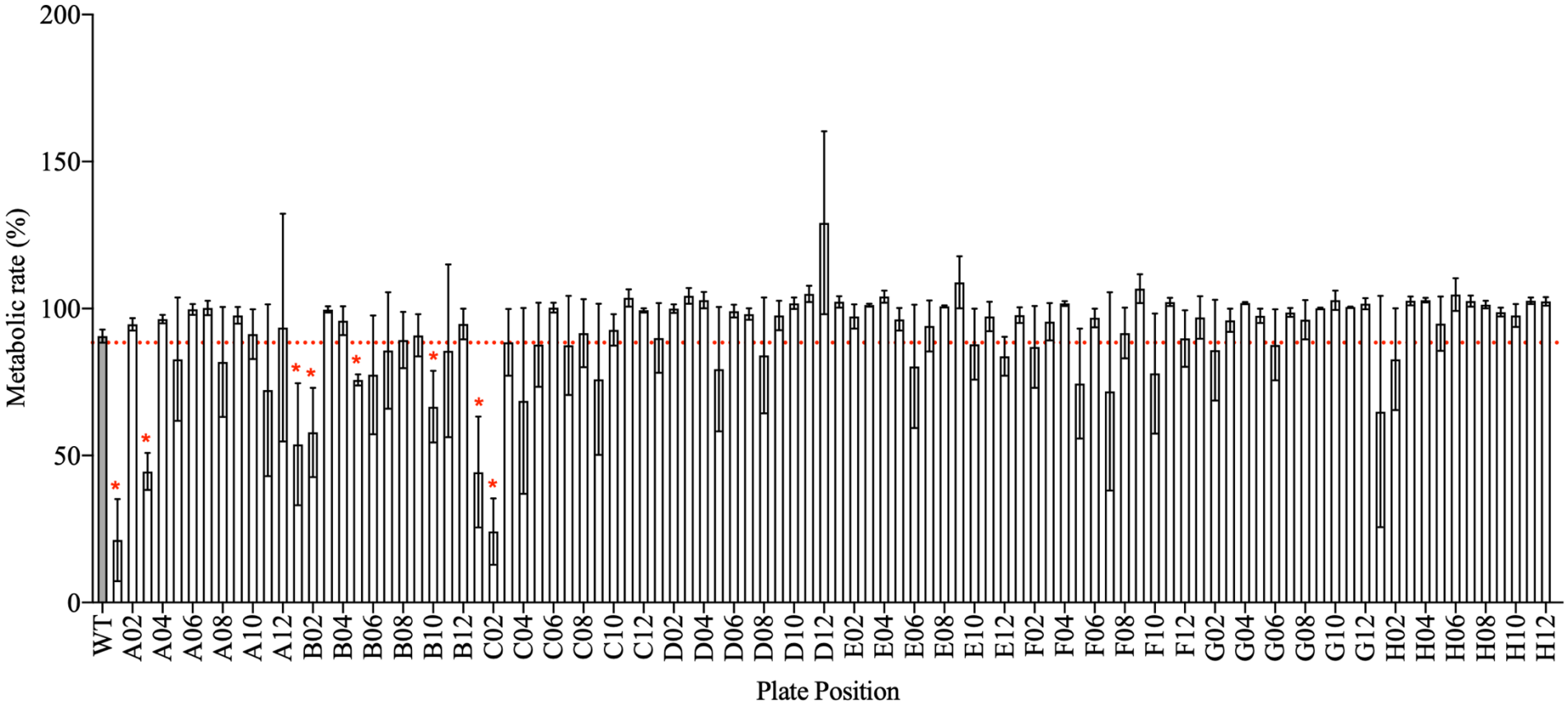


Figure 4.6: Percentage metabolic rate of GRACE™ library plate 11 mutants post-exposure to PLB-985. The GRACE™ killing assays with PLB-985 cells were performed, and the percentage (%) *C. albicans* metabolic rate was calculated based on alamarBlue™ fluorescence signals (see section 2.2.5.3); % (mean) ± SEM; *n=2*. Mutants that were chosen to be further validated with neutrophils are denoted by '*'.

GRACE™ Plate N^{O}	GRACE™ Plate Position							
1	A12	B12	E07	E08	G06	G12		
2	F12	H11	D07	G02				
7	A09	D07	F02	G03	H03	H04	H11	
11	A01	A03	B01	B02	B05	B10	C01	C02

Table 4.1: GRACE™ mutants that were chosen to be further validated with neutrophils.

Table 4.1 shows GRACE™ mutants that were susceptible to PLB-985 cells (i.e., those that had a lower percentage metabolic rate than the WT control) from plates 1, 2, 7 and 11 (except for D07, G02 from plate 1 (highlighted in yellow), where their metabolic rate was higher than WT control and >100%). These mutants were then individually validated with neutrophils once, and any mutants with a lower percentage metabolic rate than the WT control (highlighted in green) were chosen to further validate with neutrophils 3 more times.

4.2.3 FOUR GRACE^{TM} MUTANTS ARE SUSCEPTIBLE TO PRIMARY HUMAN NEUTROPHILS

The identified GRACE^{TM} mutants susceptible to PLB-985 cells (highlighted in Table 4.1) were individually screened with neutrophils once (see section 2.2.5.4). Overall, 11 out of 23 previously identified mutants were shown to be susceptible to neutrophils after the first validation (Figure 4.7). Interestingly, the 2 mutants [D07 (57.54 ± 2.86%) and G02 (39.86 ± 6.00%)] from plate 2 (that has previously been shown to have more than 100% metabolic rate after being challenged with PLB-985 cells) had a similar percentage metabolic rate to their internal WT control (49.66 ± 5.74%) after being challenged with neutrophils (Figure 4.7b).

From the initial validation, the identified 11 promising susceptible mutants were chosen for further validation with neutrophils. Figure 4.8 shows the percentage *C. albicans* metabolic rate relative to the WT control of the day. The results revealed that 9 out of 11 mutants had a mean percentage metabolic rate below 100%. However, only four of these mutants were found to be statistically significant compared to the WT control. These four mutants were A12 (62.22 ± 9.43%) from plate 2 and A01 (56.11 ± 8.53%), B10 (72.40 ± 8.46%), C02 (60.65 ± 4.26%) from plate11 (Figure 4.8). Interestingly, one mutant from plate 11 [B05 (73.15 ± 11.98%)] was found to be near significant ($p=0.054$) in comparison to the WT control. These five mutants were then chosen to be examined for their dimorphism, stress resistance and interactions with neutrophils.

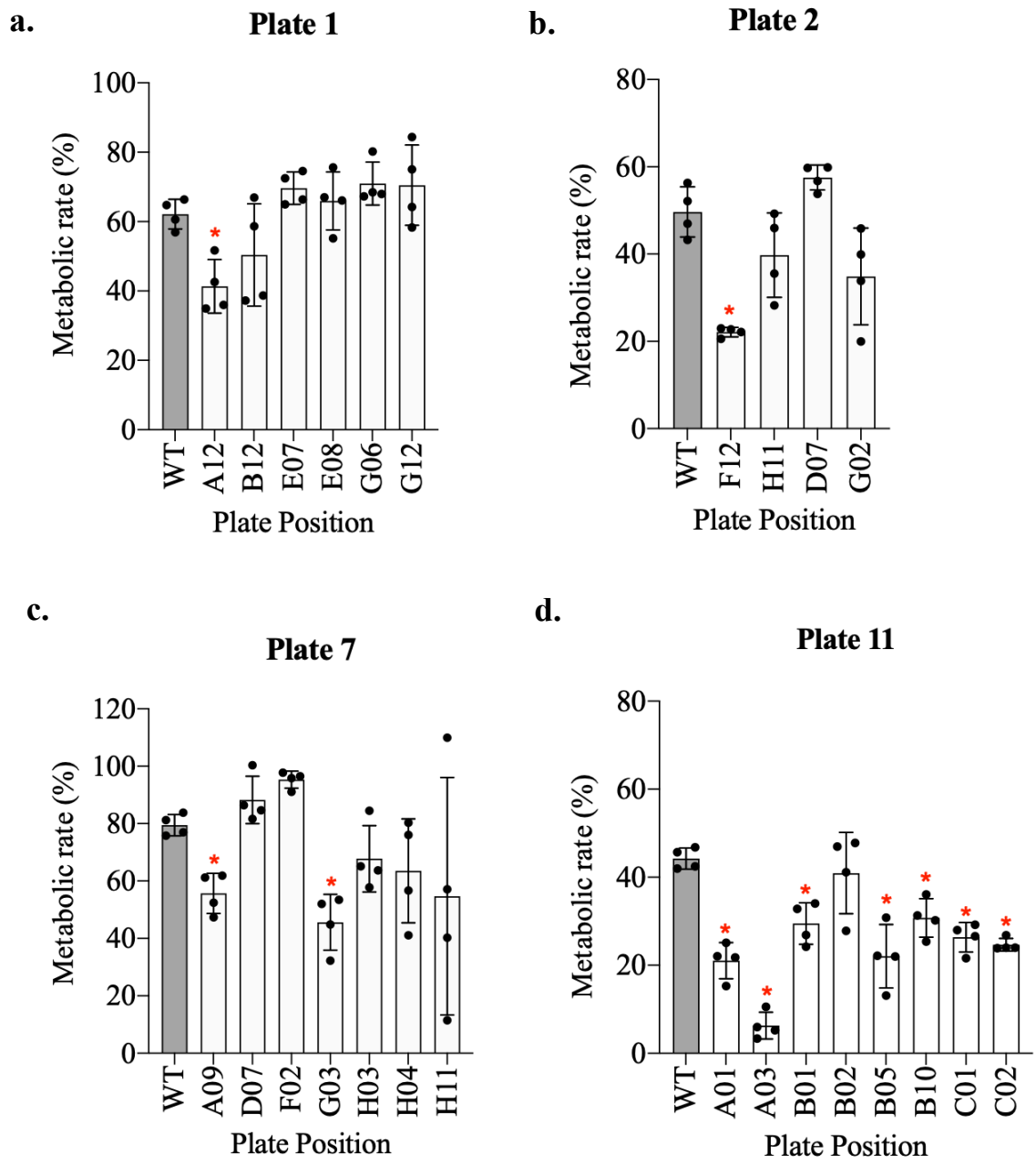


Figure 4.7: Eleven GRACE™ mutants were found to be susceptible to neutrophils after the first validation. (**a.**) Plate 1 mutants. (**b.**) Plate 2 mutants. (**c.**) Plate 7 mutants. (**d.**) Plate 11 mutants. The *C. albicans* (MOI of 5)-neutrophil killing assays were performed, and the percentage (%) *C. albicans* metabolic rate was calculated based on alamarBlue™ fluorescence signals (see section 2.2.5.4). % (mean) ± SD; *n=1* donor; each dot represents a technical replicate; mutants that were chosen to be further validated with neutrophils from 3 additional donors are denoted by '*'.

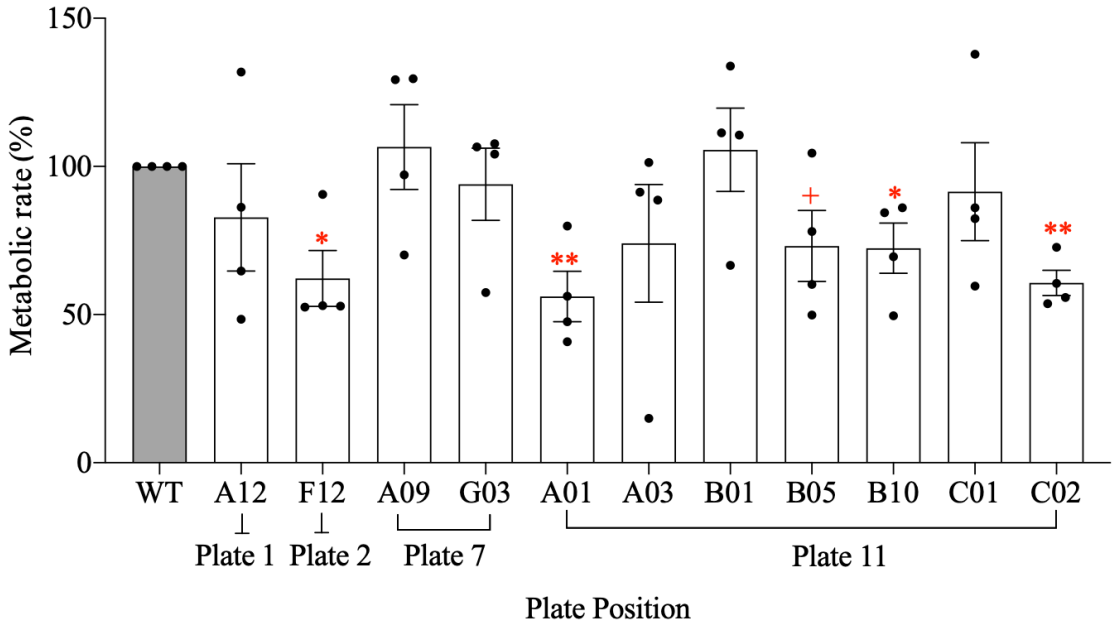


Figure 4.8: Four GRACE™ mutants were susceptible to neutrophils. *C. albicans* (MOI of 5)-neutrophil killing assays of eleven GRACE™ mutants were performed, and the percentage (%) *C. albicans* metabolic rate was calculated based on alamarBlue™ fluorescence signals (see section 2.2.5.4). % (mean) ± SEM is relative to the WT control of the day. Statistical significance was calculated by one-tailed Welch unpaired t-test; *$p<0.05$, **$p<0.01$, +$p<0.054$; $n=4$ donors.

4.2.4 GRACE^{TM} MUTANTS EXHIBIT A SIMILAR GROWTH RATE TO THE WT CONTROL.

Having identified GRACE^{TM} mutants that were susceptible to neutrophils (see Table 4.2), I next measured their growth rate in comparison to the WT control. This was to identify slow-growing phenotypes that may affect susceptibility to neutrophils. WT and mutants were incubated in YPD under doxycycline repression. The OD_{595} of the cultures were measured every 15 minutes over 24 hours (see section 2.2.2.4).

Four mutants (F12, A01, B10 and C02) had a similar maximum growth level to the WT control, while B05 had a lower maximum growth level than the WT. Interestingly, only two mutants (F12 and B10) were shown to have a similar growth rate to the WT control, while the other three mutants (A01, B05 and C02) had a lower growth rate than the control (Figure 4.9 and Table 4.2). However, it was unclear if these differences were all biologically significant, with the exception of B05, for which growth was clearly delayed.

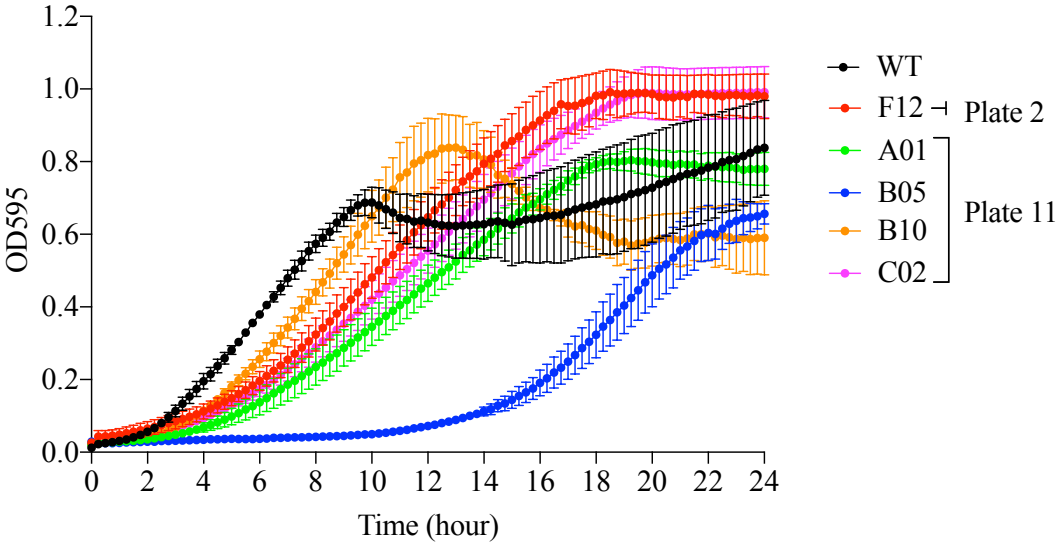


Figure 4.9: The growth curves of susceptible GRACE™ mutants. WT *C. albicans* and mutants were incubated in YPD (starting OD_{595} of 0.02) in a clear 96-well plate at 30 ºC and shaking for 24 hours. The OD_{595} of the plate was measured every 15 minutes over the incubation period (see section 2.2.2.4). Then, the OD_{595} values were plot against time. OD_{595} (mean) ± SEM; *n=3*.

4.3 DISCUSSION

This chapter aimed to identify *C. albicans* genes that are vital for the survival of neutrophil antifungal responses by screening the GRACE™ library.

Four out of 25 GRACE™ plates were initially screened with PLB-985 cells. Out of 384 mutants screened, 23 mutants (~6%) were more susceptible to PLB-985 killing than the WT control. Therefore, these 23 mutants were chosen to be further validated with neutrophils. It was expected that not all mutants would be susceptible to PLB-985 cells because only a single gene was repressed. There are many genes that contribute to the ability of *C. albicans* to evade neutrophils, and some of these genes may be redundant (Niemiec et al., 2017). Therefore, only mutants with a repressed gene that contributes the most to the evasion strategies would show up as being susceptible to PLB-985 or neutrophils.

Interestingly, over 50% of mutants screened were more resistant to PLB-985 cells than the WT control. Also, out of these "resistant" mutants, ~71% had more than 100% metabolic rate. This data suggested that some of these mutants were growing better with PLB-985 cells, which was unexpected. One explanation could be that *C. albicans* may have the ability to break down lysed-PLB-985 components and utilise these nutrients for their own growth. Another explanation could be that PLB-985 triggered *C. albicans* stress responses, causing them to increase their proliferation rate or be more metabolically active than usual, and thus a greater reduction of the alamarBlue™ reagent in PLB-985 treated wells. Also, it could be that some mutants were able to grow exponentially (i.e., remain in the log phase) after being challenged with PLB-985 cells as more cells were killed, and thus more space was available for growth. This exponential growth may not be possible in non-treated well as *C. albicans* were more

likely to be overgrown and remained in the stationary/death phase. Therefore, this resulted in a higher alamarBlue™ fluorescence signal in PLB-985 treated than non-treated wells, hence over 100% metabolic rate. Additionally, PLB-985 may be modulating filamentation, which may manifest in different metabolic rates. To investigate this observation further, 2 mutants (that had >100% metabolic rate) from GRACE™ plate 2 were chosen to be validated with neutrophils (along with the 23 susceptible mutants).

After the initial validation with neutrophils, 11 out of 25 mutants were more susceptible to neutrophils than the WT control. Interestingly, the two previously chosen "resistant" mutants from GRACE™ plate 2 showed similar susceptibility to neutrophils to the WT control. This evidence coupled with an unusually high number of mutants with >100% metabolic rate suggested that the resulting mutants with over 100% metabolic rate were likely to have been due to experimental artefacts. However, this statement is not entirely conclusive as only 2 mutants (out of >100 mutants) were validated with neutrophils. Due to large scale screening, it was expected to have some false-positive results. Thus, the two chosen mutants might have been those false positives. Not validating all previously identified resistant mutants made it impossible to state if the results were purely due to experimental artefacts. It also means that I might have missed mutants that are, in fact, resistant to neutrophils. In addition, neutrophils were slightly more potent at killing *C. albicans* compared to PLB-985 cells (Chapter 3), and this could perhaps explain the changes in *C. albicans* survival rate.

After three additional validations with neutrophils, 5 mutants (Table 4.2) with the following repressed genes were found to be susceptible to neutrophils: *BRR2, TAF7, NOC3, TFB3, SMT3* with approximately 38%, 44%, 27%, 28% and 29% reduction in cell viability relative to the CaSS1 control, respectively. Therefore, this suggested that these genes may play important

roles in *C. albicans* anti-neutrophil antifungal responses. Most of the identified genes' functions are still not well characterised and how these mutants interact with neutrophils is unknown. The next step was to study these 5 mutants' interactions with neutrophils and characterise their functions. Out of these five mutants, *noc3* was the only mutant that was borderline significant in the killing assay ($p<0.053$). Even though it was statistically near significance, it may have an important biological function. Therefore, I chose to investigate its function further with the other 4 mutants.

The growth curves revealed that all mutants, except the *noc3* mutant, exhibited similar trends to the WT control with a relatively similar growth rate and max growth. This suggested that their growth rates did not affect their susceptibility to neutrophils. However, the *noc3* mutant appeared to have a delay in growth with a slower growth rate than the WT control. This is consistent with previous literature where poor growth was observed in *NOC3* deficient *S. cerevisiae* compared to its WT control (Milkereit et al., 2001, Zhang et al., 2002). *NOC3* has also been described as an essential gene for cell growth (Milkereit et al., 2001). This implied that the *noc3* mutant growth defect could affect its susceptibility to neutrophils. Nevertheless, the *noc3* mutant growth defect may not be the sole factor for its susceptibility to neutrophils as each mutant was individually diluted to the MOI of 5 before co-incubating with neutrophils. Thus, this would have taken poor growth into consideration. It is also important to consider that these mutants were grown in YPD media at 30^{o}C (see section 2.2.2.4). This is a different condition to the killing assay, where mutants were grown in RPMI-1640 at 37^{o}C in a static incubator while encountering neutrophils. Therefore, the growth curves may not be a true reflection of how the mutants were growing. So, if I were to do the experiment again, I would grow the mutants in RPMI-1640 at 37^{o}C while measuring their OD_{595} to have a more accurate representation.

THE PREDICTED FUNCTIONS OF THE REPRESSED GENES USING THE GRACE™ SYSTEM							
Plate N°	Plate Position	Open reading frame (ORF)	Percentage metabolic rate relative to WT, % ± SEM	Growth rate, OD_{565}/hr ± SEM	Max growth, OD_{565} ± SEM	Repressed gene	Predicted roles of the encoded products
25	H4	WT (CaSS1)	100.00 ± 0.00	0.095 ± 0.010	0.838 ± 0.130	N/A	N/A
2	F12	orf19.3098	62.22 ± 9.43	0.081 ± 0.005	0.991 ± 0.060	*BRR2*	RNA helicase protein required for pre-mRNA splicing and unwinding of the U4/U6 snRNA duplex
11	A01	orf19.1574	56.12 ± 8.53	0.060 ± 0.004	0.804 ± 0.025	*TAF7*	Subunit of the TAFIID complex involved in RNA polymerase II activation
	B05	orf19.7197	73.15 ± 11.98	0.070 ± 0.003	0.657 ± 0.033	*NOC3*	Protein involved in 60S ribosomal subunit biogenesis

THE PREDICTED FUNCTIONS OF THE REPRESSED GENES USING THE GRACE™ SYSTEM (continued)							
Plate Nº	Plate Position	Open reading frame (ORF)	Percentage metabolic rate relative to WT, % ± SEM	Growth rate, OD_{565}/hr ± SEM	Max growth, OD_{565} ± SEM	Repressed gene	Predicted roles of the encoded products
11	B10	orf19.567	72.40 ± 8.46	0.098 ± 0.016	0.838 ± 0.060	*TFB3*	Component of RNA polymerase II transcription initiation TFIIH (factor b); C3HC4 zinc finger transcription factor
	C02	orf19.670	60.65± 4.26	0.070 ± 0.006	0.991 ± 0.067	*SMT3*	Ubiquitin-related protein (SUMO)

Table 4.2: The predicted functions of the repressed genes using the GRACE™ system. The table summarises the data collected for the mutants that were shown to be susceptible to neutrophils. It also shows their genetic information and the predicted functions of these genes. Some of these mutants' information [i.e., their open reading frame (ORF), repressed genes and their predicted functions] were obtained from the GRACE™ library supplier (Merck & Co.) database and the Candida Genome Database (http://www.candidagenome.org) (Skrzypek et al., 2017).

Despite the novel discoveries, there were many limitations to my experiments that should be considered. One of the significant limitations was not being able to add the same cell number of mutants across the 96-well plate. Due to a high number of mutants per plate, diluting each mutant to the exact MOI of 5 when doing the GRACE^{TM} library screen was not possible. Thus, the number of cells added to the wells was based on estimation (see section 2.2.2.2). Many factors can influence this estimation. For example, not all mutants had the same growth rates (Supplementary figure 2), meaning that more cells would have been added to the wells of mutants with a faster growth rate than those with a slower growth rate. Another influencing factor was the number of the cells that were initially inoculated in an overnight culture. This may not significantly affect fast-growing mutants' cell numbers, but it certainly would affect the mutants with a slow growth rate. It is also important to point out that I did not consider the morphology state of the mutants when doing the MOI estimation because it was impossible to do so. This means that I might have underestimated the number of mutants locked in a hyphal phase as they would not have been well suspended in solution and tend to clump together. A study suggested that using dry mass (correlated to cell surface area) instead of MOI number might be a better method to study the interactions between *C. albicans* and neutrophils as it accounted for cells with growth defects (i.e., cells that locked in either yeast or hyphal phase) (Hosseinzadeh and Urban, 2013). Also, there are many other ways *C. albicans* interacts with or responds to neutrophils (as mentioned in Chapter 1) that are driven by intracellular signalling, which is mainly influenced by cell number and not necessarily by the cell surface area. Therefore, using the MOI number might be the most appropriate method to study the interaction between *C. albicans* and neutrophils.

The MOI number is a crucial factor that influences the susceptibility of *C. albicans* to PLB-985 or neutrophils, as shown in chapter 3. This means that some mutants may have been

susceptible to PLB-985 purely because of having a lower MOI number. The opposite reasoning could also be applied to some resistant mutants. This may help to explain why over 50% of all mutants screened were shown to be resistant to PLB-985. Similarly, because too many cells were added to the wells, overpopulation may cause resorufin to reversibly reduce to the non-fluorescent dihydroresorufin (çakır and Arslan, 2010, Chen et al., 2015), as explained in chapter 3 (see section 3.4). This is likely to be the case with non-PLB-985 treated wells, as no cells were killed. Therefore, overpopulation is likely to have occurred, especially when many cells were added in the beginning. This could explain why so many mutants (over 36% of all mutants screened) had >100% metabolic rate.

Due to time constraints, I failed to thoroughly validate all 23 mutants that had been identified from the GRACE™ library screen with neutrophils. For example, an initial screen with neutrophils from one donor was not an ideal decision as this does not consider neutrophil genetic variations between different donors or differences in neutrophil subpopulations (Abbas et al., 2018, Naranbhai et al., 2015, Ng et al., 2019, Nibali et al., 2010, Treffers et al., 2018). For example, if a "healthy" donor had recently experienced an infection or suffered from inflammatory conditions, they may have had a greater immature neutrophil population in their circulation (Carissimo et al., 2020, Carmona-Rivera and Kaplan, 2013, Mortaz et al., 2018, Seebach et al., 1997, Silvestre-Roig et al., 2019). Therefore, the isolated neutrophils may not have been efficient at killing *C. albicans*. It is possible that some "healthy" donors may have been infected with COVID-19 but were asymptomatic, which could also affect the ratio of neutrophil subsets (Carissimo et al., 2020). The variation in neutrophil responses against *C. albicans* can be seen in the neutrophil optimisation data (Figure 3.4) in chapter 3 and GRACE™ mutant validation data (Figure 4.8). Therefore, there is a need to repeat the validation experiment several times with different donors to have more representative and

meaningful results. Also, the Welch unpaired t-test used in Figure 4.8 may not be the most powerful choice of statistical testing as it could introduce type 1 error. A more powerful choice of test would be one-way ANOVA. However, the WT and mutants did not have the same variance despite them being normally distributed. Therefore, the Welch unpaired t-test was the most suitable choice for statistical testing. Perhaps, having a larger sample size (i.e., more biological repeats) would allow more powerful statistical testing to be performed.

One major downfall of the screen was that it failed to identify mutants that were resistant to neutrophils (i.e., mutants lacking genes that caused them to be more vulnerable to neutrophil killing) despite over 50% of all mutants screened having greater metabolic activity than WT. It is likely that most of these mutants were false positives. This could also indicate that the optimised killing assay method was not sensitive enough to detect resistant mutants. If I have more time, I would optimise my killing assay by experimenting with different MOI numbers (i.e., between MOI of 1 and 5) and different alamarBlue™ incubation periods to better detect both directional changes. Also, it is important to continue to screen the rest of the GRACE™ library to reveal novel genes that may be important in anti-neutrophil antifungal responses.

Finally, my killing assay does not measure actual killing of *C. albicans* as the alamarBlue™ fluorescence signal is correlated to cell metabolic activity, which can be influenced by many factors (as mentioned above). This means that it does not tell us how many *C. albicans* cells survived after being challenged with PLB-985 or neutrophils. If time were not a limiting factor, I would further validate the five mutants with a more sensitive and specific assay, such as the colony-forming unit (CFU)-based killing assay. Nevertheless, the alamarBlue™-based killing assay is an excellent method for large-scale screening, like the GRACE™ library, where it is impossible to perform a CFU-based killing assay.

4.4 CONCLUSION

From the data presented in this chapter, I discovered five promising *C. albicans* mutants from the GRACE^{TM} library that were susceptible to primary human neutrophil killing. Therefore, their repressed genes may play a vital role in escaping from neutrophil antifungal responses. My next step was to characterise these mutants' functions and their interactions with neutrophils.

CHAPTER 5: CHARACTERISING SUSCEPTIBLE GRACE^{TM} MUTANTS

5.1 INTRODUCTION

Like many pathogens, *C. albicans* may evolve and mutate to a more immune resistant strain that has the capability to employ novel mechanisms to overcome neutrophil antifungal responses, such as altering its stress response pathways, ROS neutralisation and morphology to avoid phagocytosis and NETosis (Barelle et al., 2006, Branzk et al., 2014, Ermert et al., 2013, Martchenko et al., 2004, Miramon et al., 2012). By identifying these mutated genes or their functions, it could be possible to understand the anti-neutrophil antifungal response mechanisms and ultimately propose new therapeutic targets for *C. albicans* infections, such as candidemia.

The research aim of this chapter was to characterise the five GRACE^{TM} mutants which have been previously shown to be susceptible to neutrophil killing (as summarised in Table 4.2).

This chapter research objectives were to assess the mutants':

- Ability to form somatic hyphae
- Susceptibility to hydrogen peroxide
- Susceptibility to cell wall stressors
- Ability to induce neutrophil reactive oxygen species
- Ability to induce neutrophil extracellular traps

5.2 RESULTS

5.2.1 GRACE™ MUTANTS ARE PROFICIENT IN MORPHOLOGY SWITCHING

To examine the GRACE™ mutants' ability to form somatic hyphae, cells were grown in YPD media with 10% FCS for 2 hours. The morphology index (MI) score, ranging from 0 to 5, was calculated to quantify the DIC images (see section 2.2.6). Cells with an MI score closer to 1 are considered to be true yeast cells, while an MI score closer to 4 is considered to be true hyphal cells (Merson-Davies and Odds, 1989). I included a hyperfilamentous mutant, *tup1Δ/Δ*, as a positive control.

All *C. albicans* strains appeared to have a rounded morphology with MI scores that were closer to a true yeast cell in the absence of FCS and at 30 ºC, apart from the *tup1Δ/Δ* mutant, which had a more elongated morphology and the MI score closer to a true hyphal cell (Figure 5.1 and Table 5.1). Interestingly, some cells of the *noc3* mutant seemed to have an elongated shape in the absence of FCS at 30 ºC (Figure 5.1). Also, the MI score of the *noc3* mutant appeared to be slightly closer to an MI score of a true hyphal cell compared to its WT control and other four GRACE™ mutants in the absence of FCS at 30 ºC (Table 5.1). When *C. albicans* were incubated with FCS at 37 ºC, there were increases in the number of cells with elongated morphology with MI scores closer to the true hyphal cells, except from the *tup1Δ/Δ* mutant and *noc3* mutant, where there were negligible changes in the MI scores (Table 5.1).

Overall, the DIC images and MI scores showed that, to a certain extent, all GRACE^{TM} mutants had the ability to switch on filamentous growth. However, four out of five mutants (*brr2*, *noc3*, *tfb3*, *smt3*) seemed to have slightly lower capability to switch on hyphal growth compared to their WT control, while the *taf7* mutant had a similar capability to the WT control.

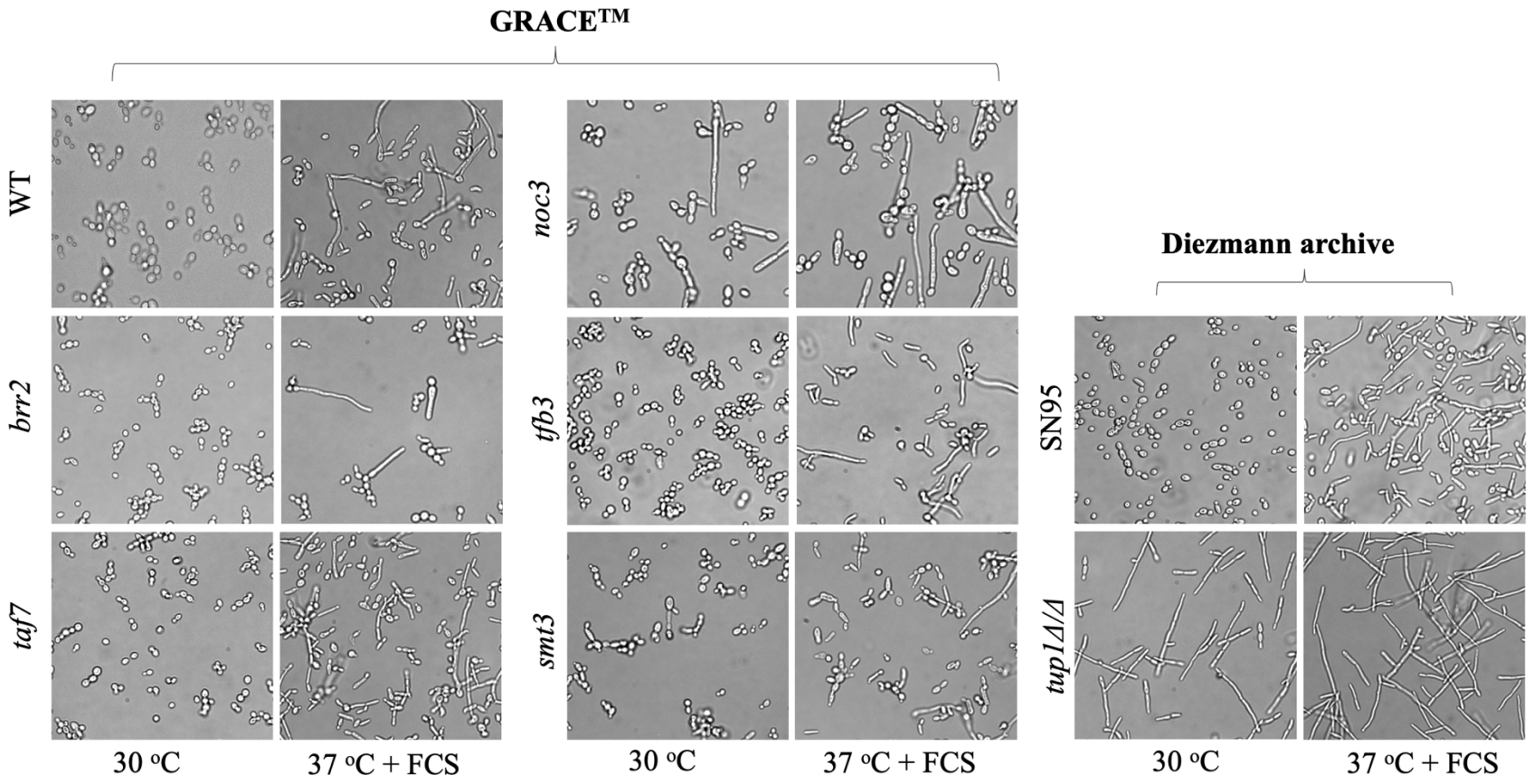


Figure 5.1: GRACE™ mutant filamentation induced by FCS. DIC images of the GRACE™ mutants and their WT control and a positive control (*tup1Δ/Δ)* from the Diezmann archive. After 4 hours of subculturing, *C. albicans* was grown in YPD media with 10% FCS for 2 hours at 37 °C and 200 rpm (see section 2.2.6). DIC images were taken using Leica DMI6000 wide-field microscopy before and after FCS incubation. Representative images of each mutant taken at 40X magnification (oil immersion) are shown. *n=1*.

Source	Mutant	Morphology Index (MI ± SD)	
		30 ºC	**10% FCS, 37 ºC**
GRACE™	WT	2.06 ± 0.32	3.59 ± 0.32
	brr2	2.15 ± 0.46	2.80 ± 0.77
	taf7	2.13 ± 0.24	3.23 ± 0.71
	noc3	2.56 ± 0.92	2.82 ± 0.85
	tfb3	2.09 ± 0.34	2.97 ± 0.71
	smt3	2.11 ± 0.34	2.78 ± 0.51
Diezmann Archive	SN95	1.93 ± 0.23	3.68 ± 0.83
	tup1Δ/Δ	3.80 ± 0.64	3.78 ± 0.61

Table 5.1: GRACE™ mutants can form somatic hyphae. After 4 hours of subculturing, *C. albicans* was grown in YPD media with 10% FCS for 2 hours at 37 ºC and 200 rpm (see section 2.2.6). Then, the morphology index (MI) score of each *C. albicans* was calculated based on DIC images. MI ± SD, *n=1*. MI score closer to 1 is considered true yeast cells, while MI closer to 4 is considered true hyphal cells.

5.2.2 THE GRACE^{TM} MUTANT SURVIVAL RATE POST-EXPOSURE TO H_2O_2

Hydrogen peroxide (H_2O_2) is one of the intermediate products of the neutrophil oxidative burst. *C. albicans* can reduce lethal ROS products such as superoxide into a less reactive H_2O_2, which in turn can be oxidised into a deadly hypochlorous acid (HOCL) by neutrophil's MPO (Amulic et al., 2012, Jamieson et al., 1996, Kobayashi et al., 2002, Segal, 2005). Although H_2O_2 is less deadly than superoxide and HOCL, it is still toxic to the cells. Therefore, some *C. albicans* mutants may not be able to tolerate H_2O_2, making them susceptible to neutrophil killing. To examine the GRACE^{TM} mutants' susceptibility to H_2O_2, cells were co-incubated with 0.1 mM H_2O_2 for 1 hour, and their survival rate was calculated based on CFU counts (see section 2.2.7).

The Hog1 pathway plays a crucial role in *C. albicans* resistance to osmotic and oxidative stresses, and *C. albicans* lacking *HOG1* was found to be susceptible to H_2O_2 (Alonso-Monge et al., 2003, Ernst and Pla, 2011, Haghnazari and Heyer, 2004). Therefore, I included the *hog1Δ/Δ* mutant as a negative control. The data showed that the negative control (64.11 ± 4.90%) had a lower survival rate than its SN95 parental control (87.77 ± 6.99%) (Figure 5.2). However, this difference was not statistically significant. This suggested that the assay failed to work as the *hog1Δ/Δ* mutant was previously optimised to be highly susceptible to H_2O_2 compared to the SN95 (data not shown, optimised by the Diezmann lab). Therefore, no definite conclusion could be drawn from this figure, which means there is a need to repeat the experiment.

The H_2O_2 assay showed that three GRACE^{TM} mutants [*taf7* (73.35 ± 11.12%), *tfb3* (80.03 ± 17.74%), *smt3* (87.77 ± 6.99%)] had a similar survival rate compared to the WT control (81.89

± 9.91%), while the other one had a lower survival rate [*brr2* (58.57 ± 4.98%)] (Figure 5.2). The result of the *noc3* mutant was excluded as no growth was detected in both H_2O_2 treated and untreated groups. Also, the survival difference between the *brr2* mutant and its WT control was not statically significant. This suggested that all GRACE mutants had the same susceptibility to H_2O_2 as the WT control.

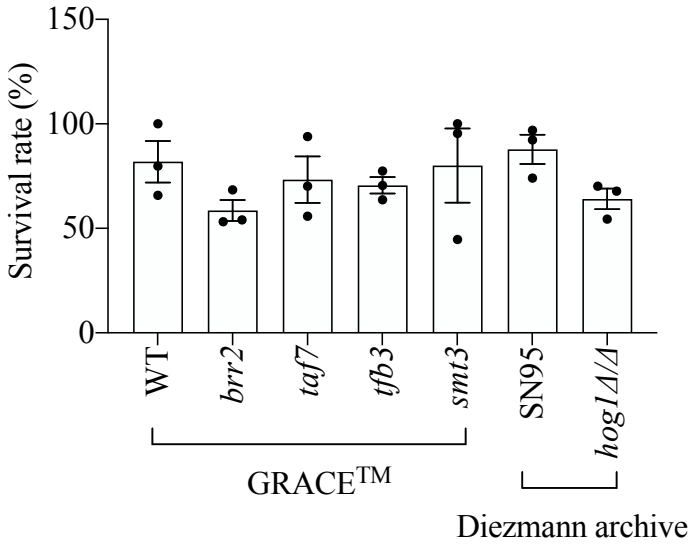


Figure 5.2: The GRACE™ mutant survival rate post-exposure to H_2O_2. *C. albicans* - H_2O_2 stress assay was performed, and the survival rate (%) was calculated based on CFU counts (see section 2.2.7). % (mean) ± SEM, n=*3*.

5.2.3 GRACE™ MUTANTS ARE SUSCEPTIBLE TO CELL WALL STRESSORS

To assess cell wall integrity, *C. albicans* were spotted onto YPD agar plates containing either CFW or Congo Red reagents and incubated at 37 ºC for 2 days (see section 2.2.8). CFW and Congo Red interfere with the assembly of the fungal cell wall and thus weaken the cell wall by targeting chitin and inhibiting enzymes responsible for the binding of chitin to β-glucans (Ram and Klis, 2006, Roncero and Durán, 1985). Mutants with low cell wall integrity and those with defective cell wall stress responses are expected to be susceptible to CFW and Congo Red reagents.

Protein kinase C (PKC) and MAPK pathways are involved in *C. albicans* cell wall integrity and biogenesis (Csank et al., 1998, Ernst and Pla, 2011). *C. albicans* cells with defective PKC and MAPK pathways, such as those lacking *EFG1, CPH1, MKK2, PKC1* were found to be susceptible, and those lacking *HOG1* were found to be resistant to CR and CFW (Eisman et al., 2006, Haghnazari and Heyer, 2004, Navarro-García et al., 2005, Román et al., 2015). Therefore, the following controls were included: negative controls (*efg1Δ/Δ cph1Δ/Δ*, *mkk2Δ/Δ* and *pkc1Δ/Δ*) and a positive control (*hog1Δ/Δ*).

The images revealed that two negative controls (*mkk2Δ/Δ* and *pkc1Δ/Δ*) had poor growth compared to their SN95 parental control on both CFW and Congo Red (Figure 5.3). The images also illustrated that the positive control had slightly better growth on the CFW plate and unexpectedly slightly poorer growth on the Congo Red plate. Therefore, only limited conclusions could be drawn from this experiment.

All *C. albicans* strains that grew on both CFW and Congo Red plates exhibited lower levels of growth than on the YPD only plate (Figure 5.3). This suggested that all *C. albicans* strains were somewhat susceptible to CFW and Congo Red. The images also revealed that two out of five mutants (*brr2* and *smt3*) seemed to have poorer growth on the CFW and Congo Red agar plates than their WT control. Meanwhile, the *taf7* mutant seemed to grow slightly better than its WT control on both CFW and Congo Red agar plates. Interestingly, the *tfb3* mutant seemed to grow slightly better on the CFW plate and poorer on the Congo Red plate than its WT control.

The data for *noc3,* CAI4 and *efg1Δ/Δ cph1Δ/Δ* strains were excluded. This is because the *noc3* mutant did not grow robustly on the YPD only plate. Meanwhile, the *efg1Δ/Δ cph1Δ/Δ* mutant grew too well compared to its CAI4 parental control on the YPD only plate. Therefore, it was quite difficult to judge their susceptibility to Congo Red and CFW reagents by eye, and thus no comparisons were made.

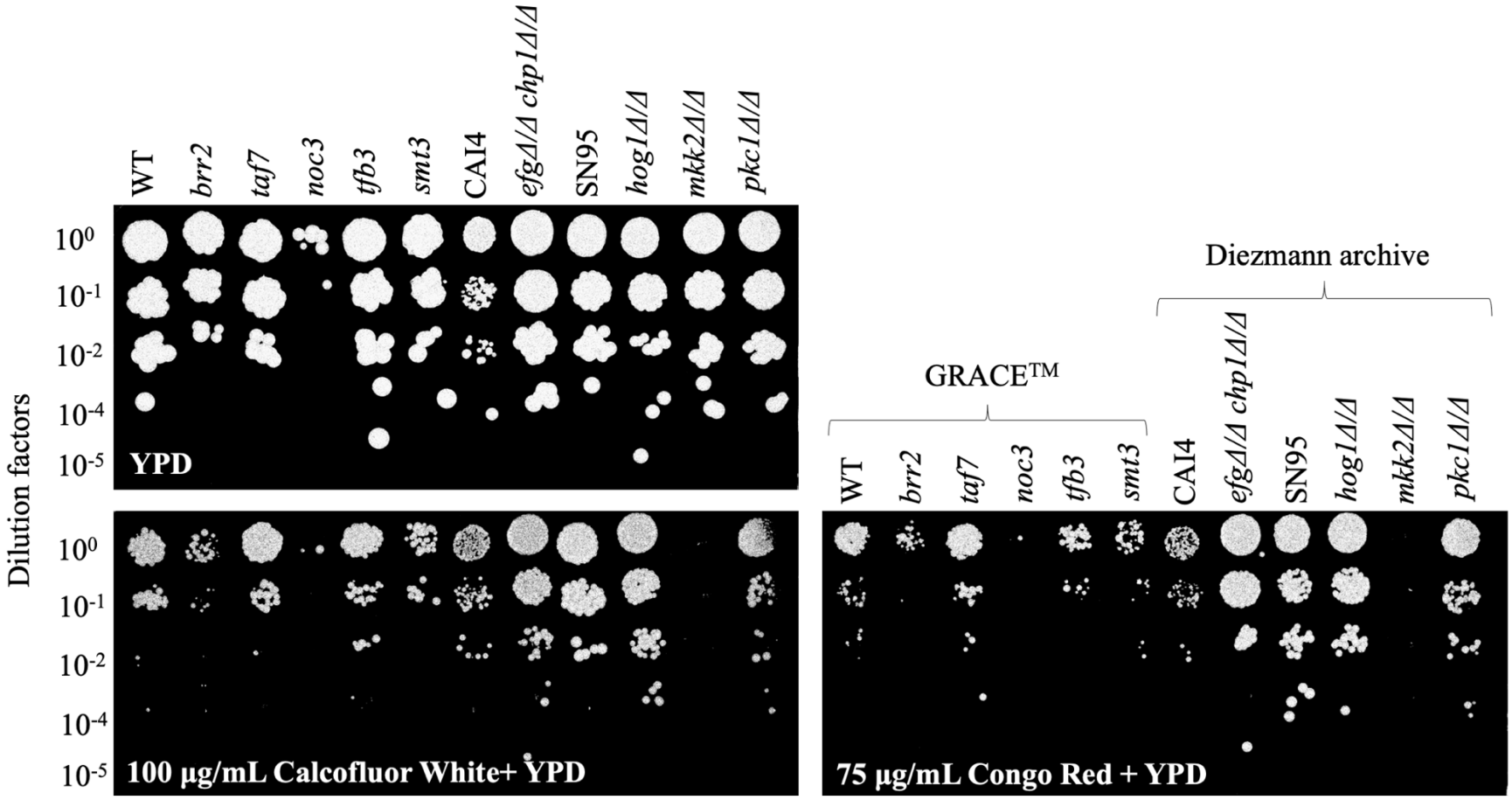


Figure 5.3: The GRACE™ mutants are susceptible to Congo Red and CFW. Ten-fold serial dilutions of *C. albicans* were spotted onto YPD agar plates containing either 100 µg/mL CFW or 75 µg/mL Congo Red reagents and incubated at 37 ºC for 48 hours (see section 2.2.8). *n=1*.

5.2.4 GRACE™ MUTANTS ARE STRONG INDUCERS OF NEUTROPHIL OXIDATIVE BURST

Reactive oxygen species (ROS) production is one of the main neutrophil antifungal responses against *C. albicans* (Jamieson et al., 1996, Segal, 2005). The respiratory burst using the luminol method was performed to assess how the GRACE™ mutants affect neutrophil ROS production (see section 2.2.9).

I first compared the following *C. albicans* mutants from the Diezmann archive to analyse how filamentation ability affects the oxidative burst: a hyperfilamentous mutant (*tup1Δ/Δ*) and a yeast-locked mutant (*efg1Δ/Δ cph1Δ/Δ*). The kinetic curves showed that the *efg1Δ/Δ cph1Δ/Δ* mutant (14,384 ±4,603) had a considerably higher overall ROS chemiluminescence signal than its CAI4 parental strain (5,646 ± 2,816) (Figure 5.4a). Interestingly, the *tup1Δ/Δ* mutant (1,039 ± 177) had a much lower overall ROS chemiluminescence signal than its SN95 parental control (6,842 ± 3,064) (Figure 5.4a). However, it was difficult to quantify the *tup1Δ/Δ* mutant, and thus I may have added fewer cells than expected.

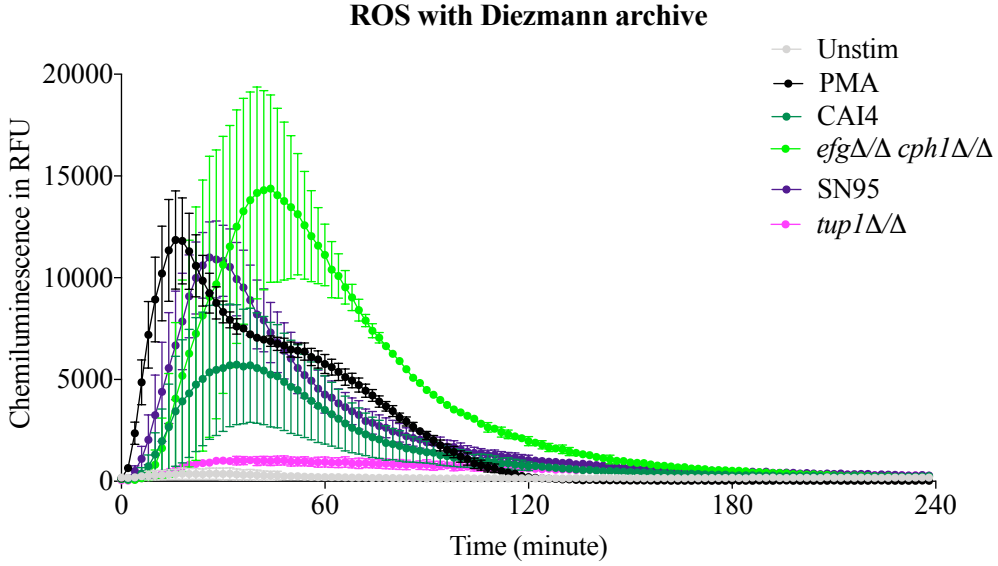
The area under the ROS curves was calculated to estimate neutrophil total ROS production (Figure 5.5). The graph also showed that the total ROS production triggered by the *efg1Δ/Δ cph1Δ/Δ* mutant (775,361 ± 211,039) was significantly higher than the CAI4 control (313,737 ± 153,480). Although there was a visible difference in the total ROS production between the *tup1Δ/Δ* mutant (79,387 ± 17,093) and the SN95 control (501,207 ± 90,363), this difference was not statically significant. Therefore, the data suggested that the yeast-lock mutant may be a potent ROS inducer, while the hyperfilamentous mutant may be a weak ROS inducer.

The kinetic curves showed that out of five GRACE^{TM} mutants, *tfb3* had the highest, and *smt3* had the lowest overall ROS chemiluminescence signal (Figure 5.4b). Three GRACE^{TM} mutants [*brr2* (9,152 ± 405), *taf7* (8,660 ± 360), *tfb3* (9,859 ± 896)] appeared to have a similar overall ROS chemiluminescence signal based on the peak values to their WT control (8,705 ± 1,239), while the other two mutants [*noc3* (6,367 ± 177) and *smt3* (5,375 ± 803)] had a far lower overall ROS chemiluminescence signal than the WT control (Figure 5.4b). It also appeared that the *taf7* mutant peaked relatively early (at 16 minutes) compared to the other mutants, which peaked at a similar time to the WT (at ~24 minutes).

Like the GRACE^{TM} mutant kinetic curves, *tfb3* triggered the highest total ROS production, while *smt3* elicited the least total ROS production. In contrast to the kinetic curves, all GRACE^{TM} mutants [*brr2* (458,388 ± 41,548), *taf7* (417,7752 ± 19,761), *noc3* (334,216 ± 4,941), *tfb3* (524,553 ± 72,462), and *smt3* (315,016 ± 58,575)] induced a similar amount of ROS production to their WT control (433,254 ± 88,321) (Figure 5.5).

Overall, the respiratory burst data suggested that the five GRACE^{TM} mutants had a similar capacity to induce neutrophil ROS production.

a.



b.

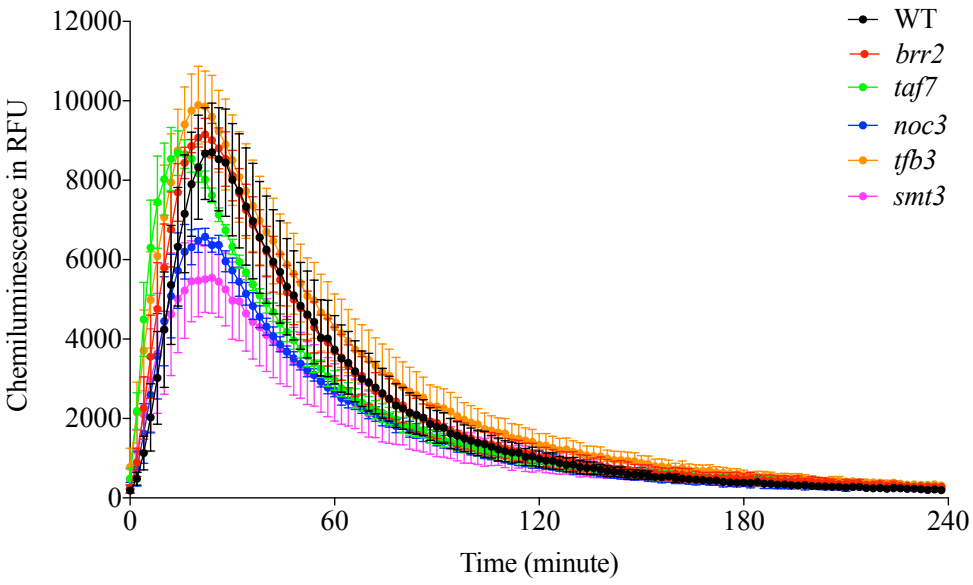


Figure 5.4: Neutrophil ROS production in response to *C. albicans* infection. (**a.**) Chemiluminescence of reactive oxygen species (ROS) with *C. albicans* mutants from the Diezmann archive. (**b.**) Chemiluminescence of ROS with GRACE™ mutants and their WT control. Chemiluminescence (mean in RFU) ± SEM. *n=3* donors. Neutrophils were challenged with *C. albicans* at an MOI of 5 (see section 2.2.9).

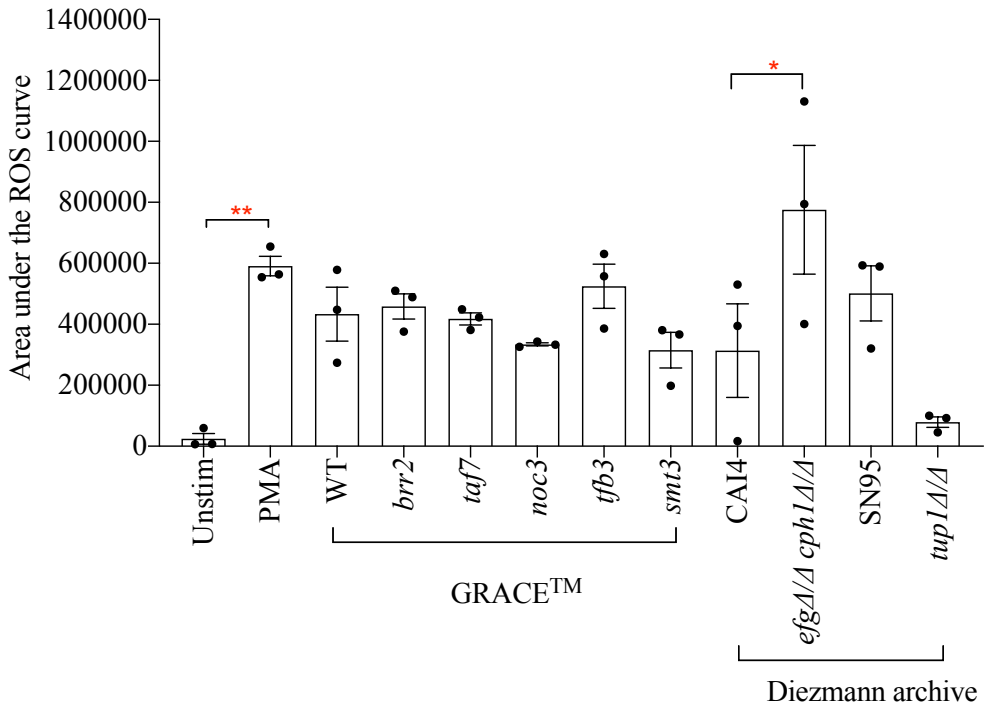


Figure 5.5: Neutrophil total ROS production in response to *C. albicans* infection. Neutrophils were challenged with *C. albicans* at an MOI of 5, and the area under the ROS curve of each *C. albicans* strain was calculated to determine total ROS production (see section 2.2.9). Area under the curve (mean) ± SEM. Statistical significance was calculated by one-way ANOVA; *$p<0.05$, **$p<0.01$; $n=3$ donors.

5.2.5 MUTANTS WITH GROWTH DEFECTS TRIGGERED LESS NETOSIS

To examine the GRACE™ mutants' ability to induce NETosis, a simple NETs assay was performed using fluorescent DNA staining dyes: Sytox™ Orange (detects extracellular DNA, which is assumed to indicate NETs) and Syto™ Green (detects intracellular DNA). NET images were then taken by the EVOS FL Cell Imaging System (see section 2.2.10). Then, the number of NETs (red areas in Figure 5.6-5.7) per image was quantified.

I included neutrophils only condition as a negative control, where no NETs were detected (Figure 5.6, top row). To assess how filamentation ability affects NETosis, *tup1Δ/Δ* and *efg1Δ/Δ cph1Δ/Δ* mutants were included in the NETs assay. Based on NETs per image (NPI) data, the *efg1Δ/Δ cph1Δ/Δ* mutant (195 ± 12) had a significantly lower NPI than its CAI4 parental control (433 ± 20) (Figure 5.8). Unexpectedly, the *tup1Δ/Δ* mutant (123 ± 17) appeared to have a significantly lower NPI than its SN95 parental control (367 ± 57). This is likely because fewer cells of the *tup1Δ/Δ* mutant were added due to the quantification difficulty.

Figure 5.6-5.7 (top row) shows that all *C. albicans*, including the five GRACE™ mutants, were capable of inducing NETs release. Interestingly, the images show that all GRACE™ mutants exhibited filamentous growth after 4 hours of incubation in RPMI-1640 (with 2% human pooled plasma). However, four out of five GRACE™ mutants (*brr2, noc3, tfb3 and smt3*) appeared to be unable to form true hyphae compared to the WT control as some of their cells appeared to be somewhat like the morphology of *efg1Δ/Δ cph1Δ/Δ* (Figure 5.6-5.7, bottom row).

Out of five GRACE™ mutants, *tfb3* (379 ± 13) and *smt3* (379 ± 22) triggered the most, and *noc3* (107 ± 15) triggered the least NETs release, while the other two mutants [*brr2* (303 + 23) and *taf7* (285 ± 55)] induced similar NETs release levels to their WT control (286 ± 21) (Figure 5.8). However, the differences between *tfb3* and *smt3* to their WT control were not statistically significant despite visible changes in NPI. Nonetheless, there was a statistically significant difference between *noc3* and its WT control.

Overall, my data suggested that mutants with growth defects (i.e., either in yeast-locked or hyphae-locked forms) perhaps have less ability to induce NETosis.

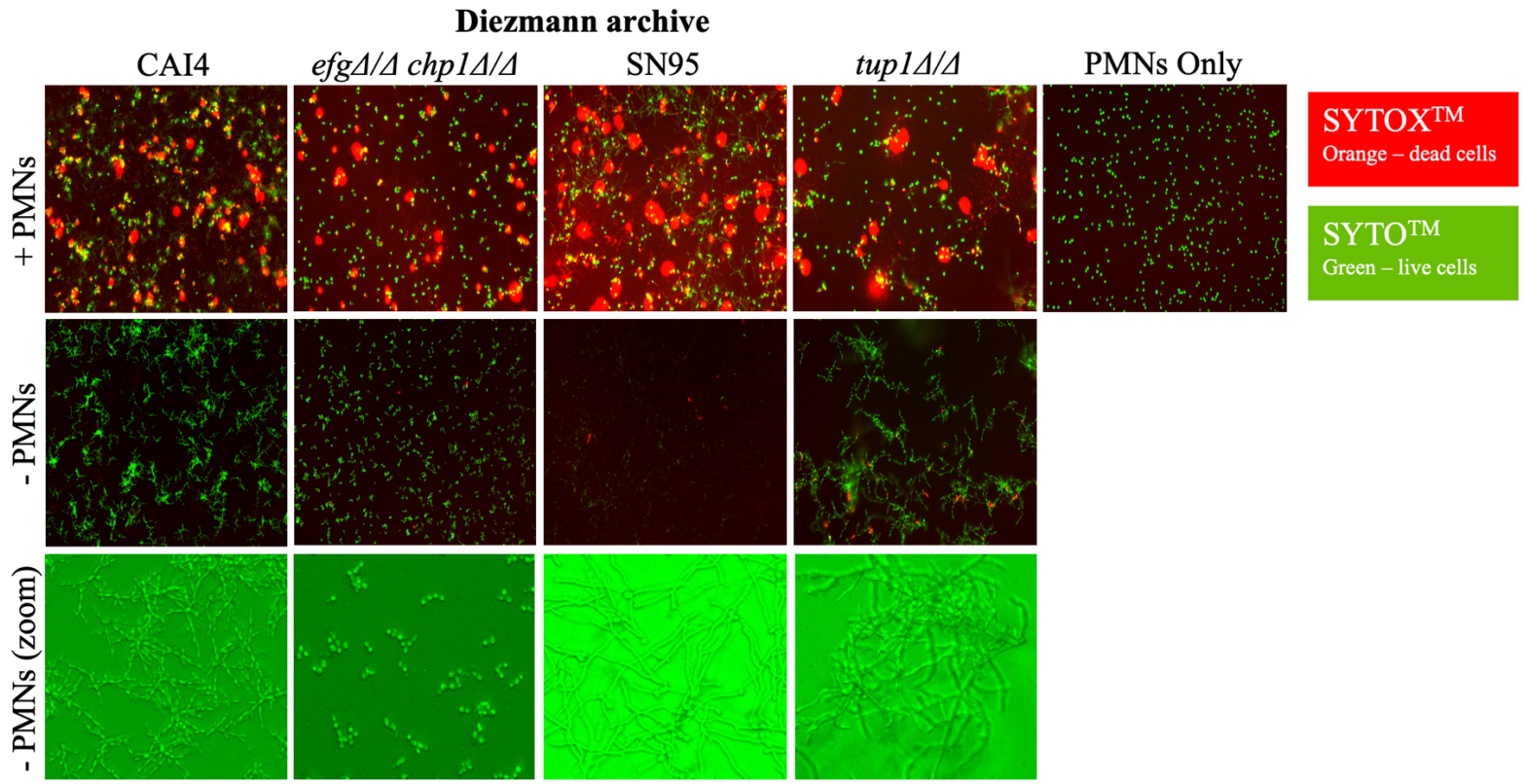


Figure 5.6: *C. albicans* is a robust NET inducer. Syto™/Sytox™ images of *C. albicans* strains from the Diezmann archive. This figure shows representative images of each mutant taken at 10X magnification, where Syto™ green (green channel) indicates live cells and Sytox™ orange (red channel) indicates dead cells. Top row: images of *C. albicans* + neutrophils (in green and red channels); Middle row: images of *C. albicans* only (in green and red channels); Bottom row: zoom-in images of *C. albicans* only (in the green channel). Neutrophils were challenged with *C. albicans* at an MOI of 5, *n=3* donors.

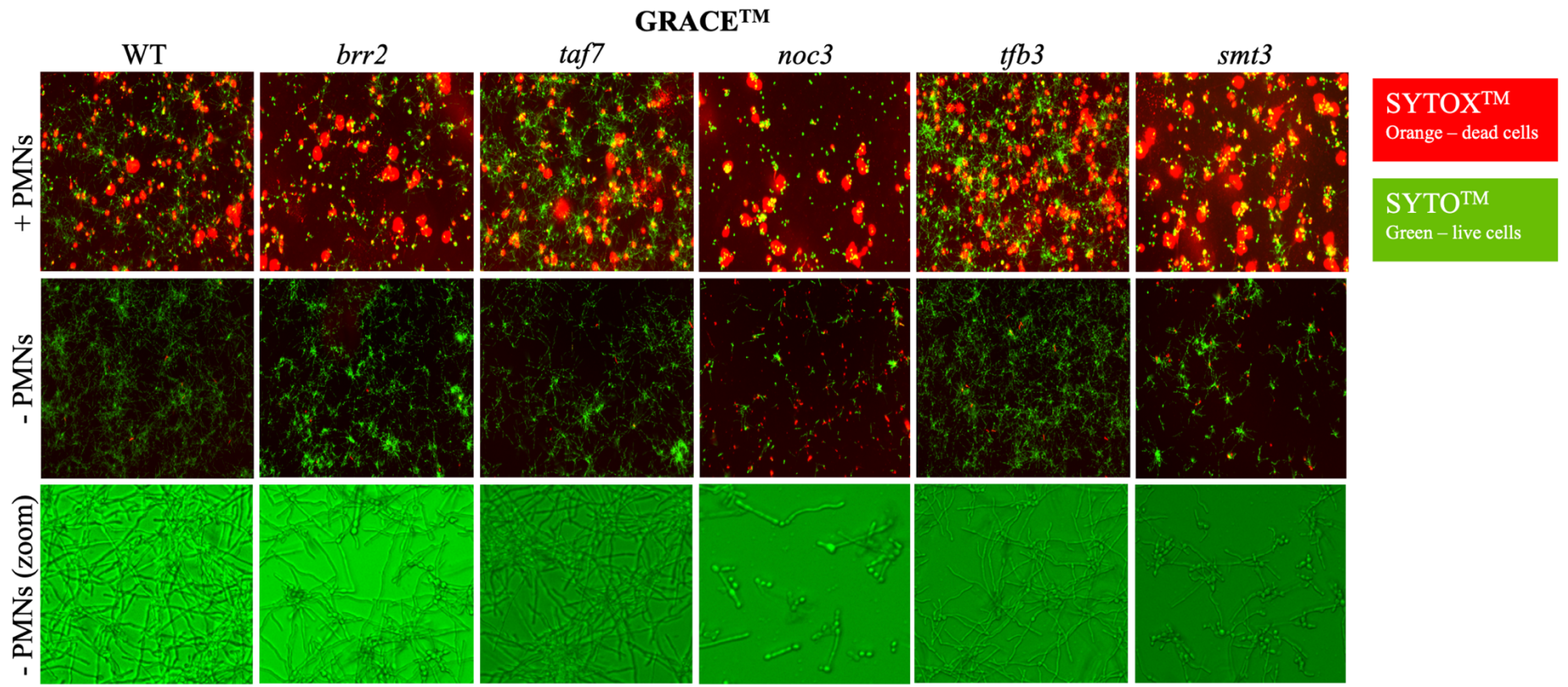


Figure 5.7: GRACE™ mutants are strong NET inducers. Syto™/Sytox™ images of GRACE™ mutants and their WT control. This figure shows representative images of each mutant taken at 10X magnification, where Syto™ green (green channel) indicates live cells and Sytox™ orange (red channel) indicates dead cells. Top row: images of *C. albicans* + neutrophils (in green and red channels); Middle row: images of *C. albicans* only (in green and red channels); Bottom row: zoom-in images of *C. albicans* only (in the green channel). Neutrophils were challenged with *C. albicans* at an MOI of 5, *n=3* human donors.

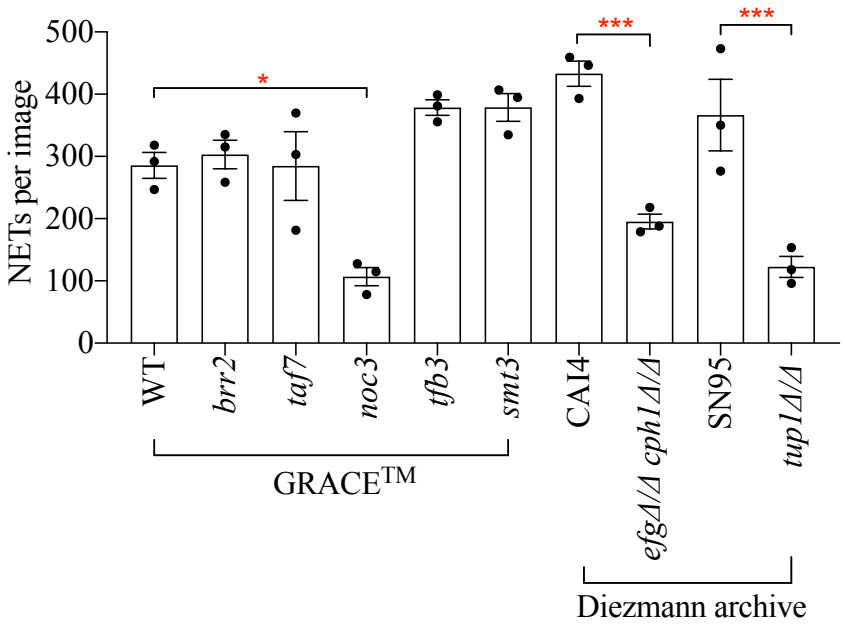


Figure 5.8: GRACE™ mutants can trigger NETosis. Neutrophils were challenged with *C. albicans* at an MOI of 5, and the number of NETs per image of each *C. albicans* strain was calculated (see section 2.2.10). NETs per image (mean) ± SEM. Statistical significance was calculated by one-way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$; $n=3$ donors.

5.3 DISCUSSION

This chapter aimed to characterise the five GRACE^{TM} mutants, (as summarised in Table 4.2) functions and their interactions with neutrophils.

The ability to switch between the yeast and the hyphal phase is one of the main virulence factors of *C. albicans* (Pappas et al., 2018, Wibawa, 2016). Mutants that lack this ability are more likely to be susceptible to neutrophil killing (Ermert et al., 2013, Jayatilake et al., 2007, Moyes et al., 2010, Naglik et al., 2011, Salvatori et al., 2018). To assess the morphogenetic diversity of the GRACE^{TM} mutants, a serum-induced filamentation assay was performed. The assay revealed that all GRACE^{TM} mutants could form somatic hyphae to a certain extent in response to FCS serum. Three GRACE^{TM} mutants (*brr2*, *tfb3*, *smt3*) appeared to be slightly less efficient at transitioning from yeast to hyphae than the WT control. Therefore, these three genes may be involved in cell morphogenesis.

The *SMT3* gene encodes a ubiquitin-like protein (SUMO), which modulates post-translational modification of many proteins (Dieckhoff et al., 2004, Ghaemmaghami et al., 2003, Johnson et al., 1997, Martin and Konopka, 2004, Skrzypek et al., 2017, Takahashi et al., 1999). A study showed that the *smt3* mutant could elongate its cell in response to FCS serum induction but failed to form a true hypha (Leach et al., 2011). This evidence is consistent with the MI score of the *smt3* mutant reported in this project. Perhaps, SUMO modifies proteins that are involved in the yeast-hypha morphogenesis.

The *BRR2* gene encodes pre-mRNA-splicing helicase, which plays an essential role in pre-mRNA splicing (Ghaemmaghami et al., 2003, Hahn et al., 2012, Maeder et al., 2009, Pena et

al., 2009, Skrzypek et al., 2017, Zhang et al., 2009). The *TFB3* gene encodes RNA polymerase II transcription factor subunit 3, which is vital for DNA transcription and repair (Faye et al., 1997, Feaver et al., 1997, Ghaemmaghami et al., 2003, Skrzypek et al., 2017). Therefore, it may be possible that the *BRR2* and *TFB3* genes products are involved in the transcription of other important genes in yeast-hypha morphogenesis.

Both before and after serum treatments, the *noc3* mutant interestingly appeared to have MI scores that are indicative of hyphal growth. This suggested that the *noc3* mutant may not have the ability to entirely switch between yeast and hyphal forms and thus implied that the *NOC3* gene may play an essential role in the yeast-hypha morphogenesis. Two studies showed that *NOC3* is an essential gene for the initiation of DNA replication and the synthesis of 60S ribosomal subunits in *S. cerevisiae* (Milkereit et al., 2001, Zhang et al., 2002). Since these two processes are important for cell maturation, the studies suggested the *NOC3* gene to be vital for fungal growth (Milkereit et al., 2001, Zhang et al., 2002). This suggestion is further supported by my growth curve data (Chapter 4, Figure 4.9), where the *noc3* mutant had the lowest growth rate and maximum growth compared to its WT and other tested GRACE^{TM} mutants. Perhaps, the inability to transition between the two morphologies and the growth defect caused the *noc3* mutant to become susceptible to neutrophil killing.

Despite the interesting findings, it is important to consider that the serum-induced filamentation assay was done in completely different conditions to the killing assay. For example, YPD media was used instead of RPMI-1640 media, and 10% FCS was used instead of 2% human pooled plasma serum. These conditions may have some influence on *C. albicans* behaviour. Maybe to have a more informative and reflective result of how *C. albicans* dimorphism affects neutrophil killing ability, the filamentation assay should be conducted in similar conditions to

the killing assay. Also, *C. albicans* mutants were only incubated with FCS for 2 hours. This means that the obtained data might not truly reflect their ability to form somatic hyphae as some of them may take longer to switch on the filamentous growth programming. Furthermore, I only performed the serum-induced filamentation assay once. Thus, more experimental repeats are needed to produce a more representative result. In addition, *C. albicans* strains, such as the *efg1Δ/Δ cph1Δ/Δ* mutant, should be included in the assay as a negative control.

The H_2O_2 assay was performed to assess the GRACE^{TM} mutants' ability to tolerate a neutrophil toxic intermediate product, H_2O_2 (Amulic et al., 2012, Jamieson et al., 1996, Kobayashi et al., 2002, Segal, 2005). The assay failed to work as there was no significant difference between the survival rate of the negative control and the WT. Despite this, there seemed to be some interesting trends among the GRACE^{TM} mutants. For example, *brr2* and *tfb3* mutants had a consistently lower survival rate than their WT control (Supplementary figure 3). This may suggest that *BRR2* and *TFB3* genes might have some contribution to H_2O_2 tolerance. However, no valid conclusions can be drawn from this experiment until repeated. The use of old H_2O_2 stock (from 2016) is likely to explain why the experiment did not work. Since H_2O_2 is inherently unstable, it is likely that I have used a lower concentration of H_2O_2 than the one stated in the method. If I were to do the experiment again, I would make sure to use the correct concentration of H_2O_2. I would also adjust the plating cell concentration for slow grower mutants like the *noc3* mutant, where no growth was detected in both H_2O_2 treated and untreated groups.

CFW and Congo Red interfere with cell wall formation by inhibiting chitin and β-(1,3)-glucan synthases (Ram and Klis, 2006, Roncero and Durán, 1985). Thus, these reagents were used to test the cell wall integrity of each GRACE^{TM} mutant. Compared to their WT control, *brr2* and

smt3 mutants appeared to be more susceptible, and *taf7* mutant seemed to be more resistant to Congo Red and CFW reagents. This suggested that *BRR2, SMT3* and *TAF7* genes might play essential functions in maintaining cell wall integrity, such as synthesising chitins and β-glucans.

A study also showed that the *smt3* mutant grew poorly in Congo Red and CFW compared to its control, which further reinforced the potentially critical role of the *SMT3* gene in cell wall damage signalling (Leach et al., 2011). The study also showed Erg13 protein to be one of the SUMOylating targets in *C. albicans*. Erg13 is a protein involved in ergosterol biosynthesis, a crucial process in maintaining the cell membrane (Leach et al., 2011, Lv et al., 2016, Servouse et al., 1984). Perhaps, cells lacking the *SMT3* gene might have a defect in the cell membrane and cell wall structures, thus making them more susceptible to Congo Red and CFW.

The *TAF7* gene encodes a transcription initiation factor TFIID subunit 7 protein, which is involved in the regulation of gene expression by RNA polymerase II (Bhattacharya et al., 2014, Kolesnikova et al., 2018, Matangkasombut et al., 2000, Sanders et al., 2002, Sanders and Weil, 2000). Although no studies were done on how *TAF7* affects cell wall synthesis, my data suggest that the *TAF7* gene may be involved in the breakdown of cell wall components as the mutant lacking *TAF7* was more resistant to CFW and Congo Red. For example, *TAF7* may enhance the transcription of genes that encode cellulase and proteases.

Interestingly, the cell wall stressors assay also showed that the *tfb3* mutant was more resistant to CFW and more susceptible to Congo Red than the WT control. As CFW and Congo Red interfere with the *C. albicans* cell wall in a similar way, it was expected that the *tfb3* mutant would have a similar susceptibility pattern to both reagents. One explanation for this difference

could be that CFW may prefer to bind to chitin than β-glucans, while Congo Red may prefer to bind to β-glucans than chitin. Therefore, if a mutant, such as *tfb3*, has more chitin and fewer β-glucans expressed on its cell wall, it might be less susceptible to CFW and more susceptible to Congo Red. A study showed that the *TFB3* gene was upregulated in *C. albicans* treated with caspofungin, a drug that works similarly to CFW and Congo Red by inhibiting β-1,3-glucan synthases (Reinoso-Martin et al., 2003). This suggested that *TFB3* may be important in the synthesis of β-1,3-glucan. For example, it perhaps enhances the transcription of β-1,3-glucan or enzymes that are involved in β-1,3-glucan synthesis. Also, this piece of evidence has somewhat supported the response of the *tfb3* mutant to Congo Red as cells lacking the *TFB3* gene would be expected to be more susceptible to cell wall stressors.

Before any definite conclusions can be made, it is important to consider some limitations of the cell wall stressor assay. Firstly, the results were not quantitative, and any analyses were done qualitatively by eye. This means that different individuals may interpret the data differently. Using the CFU-based method (similar to the H_2O_2 stress assay as described in section 2.2.7) may be a better way to conduct this experiment to avoid bias. Also, the assay does not take the growth rate of *C. albicans* into account. Therefore, it is difficult to distinguish between the cell wall susceptibility and the mutant's ability to grow. For example, faster growth mutants may look less, and slower growth mutants may look more susceptible to CFW and Congo Red than they actually are. Furthermore, the assay was conducted once, and thus more repeats are needed to confirm the finding.

ROS production is one of the main neutrophil antifungal responses when encountering *C. albicans* (Jamieson et al., 1996, Segal, 2005). The ROS kinetic curves showed that all mutants could induce ROS production. *noc3* and *smt3* mutants appeared to be weak ROS inducers as

they had the lowest ROS peaks compared to their WT and other mutants. However, the area under the kinetic curves revealed no significant difference in the total ROS production between GRACE^{TM} mutants and their WT. Interestingly, the total ROS production triggered by *smt3* appeared to be consistently lower than the total ROS production induced by the WT across each biological replicate (i.e., across different neutrophil donors), suggesting it to be a weak ROS inducer (Supplementary figure 4). Conversely, the total ROS production triggered by the *tfb3* mutant appeared to be consistently higher than the total ROS production induced by the WT across each biological replicate, suggesting it to be a potent ROS inducer (Supplementary figure 4).

It became apparent that interpreting the ROS kinetic curves and the area under the kinetic curves can lead to contradicting conclusions, such as how powerful each mutant is at inducing ROS production. This raises the question of how to define a "strong inducer". Is it by how much ROS is produced at a specific time (i.e., the peak) or how much ROS is produced over time (i.e., the area under the curve)? ROS production initiation relies on the recognition of pathogens (Nguyen et al., 2017). Therefore, mutants that induced less ROS production, such as *noc3* and *smt3* mutants, may have fewer PAMPs on their surface, making them harder to recognise. The opposite could be applied to mutants that induced more ROS production, such as the *tfb3* mutant. This further suggested that *NOC3, SMT3* and *TFB3* genes may be involved in the synthesis of PAMPs that are responsible for ROS induction. Furthermore, previous research suggested that there may be a positive correlation between ROS production and mutants (such as *sod5Δ/Δ*) that are sensitive to oxidative stress (Martchenko et al., 2004, Miramon et al., 2012). This is somewhat similar to the finding of the *tfb3* mutant, where it consistently induced a higher ROS production and had a consistently lower survival when being challenged with H_2O_2 than its WT control. This further suggested that the *TFB3* gene

may also be important in the detoxification of ROS. For example, Tfb3 possibly enhances the transcription of proteins that can neutralise ROS, such as Sods. It is worth mentioning that the ROS assay does not tell us about how susceptible the *C. albicans* strains are to ROS. For example, mutants that induce more ROS are not necessarily more susceptible than those that induce less ROS. Such conclusions can only be made from a survival assay that exposes *C. albicans* directly to ROS components, such as superoxide and H_2O_2.

To assess how different morphologies could affect ROS production, I also stimulated neutrophils with the *efg1Δ/Δ cph1Δ/Δ* double mutant and the *tup1Δ/Δ* mutant. The data revealed that the *tup1Δ/Δ* mutant induced the least amount of ROS compared to other tested strains. This finding contradicts a ROS study, where the dry mass of *C. albicans* was used to stimulate neutrophils (Hosseinzadeh and Urban, 2013). The study showed that *tup1Δ* produced a similar level of ROS production to its WT and *efg1Δ* of the same mass (Hosseinzadeh and Urban, 2013). As mentioned in chapter 4, hyperfilamentous mutants tend to clump together and are not well suspended, and thus I might not have added enough *tup1Δ/Δ* cells to trigger a robust ROS response. Perhaps using dry mass that accounted for cell surface area for this experiment might be more sensible as ROS initiation mainly relies on cell surface recognition. Furthermore, my data revealed that the *efg1Δ/Δ cph1Δ/Δ* mutant induced a significantly higher ROS production than its WT. This finding somewhat contradicts a macrophage study, where *efg1Δ/Δ* induced less ROS production, while *efg1Δ* induced more ROS production than its WT control (Zavrel et al., 2012). Conversely, the dry mass study showed that ROS induction by the *efg1Δ* mutant was overall slightly lower than its WT control (Hosseinzadeh and Urban, 2013). As three different yeast-locked mutants triggered different ROS responses and a yeast-locked mutant can trigger ROS as efficiently as a hyphae-locked mutant, it seems that the morphology of *C. albicans* is not a sole factor and may not play a significant role in ROS production. It is

also clear that more investigations are needed to clarify why different mutants of the same morphology-lock trigger ROS production differently. Furthermore, a separate assay for intracellular and extracellular ROS production should be conducted in the future as they may give more specific information on how each mutant affects neutrophil ROS production.

NETosis is another important pathogen killing mechanism of neutrophils (Amulic and Sollberger, 2019, Branzk et al., 2014, Papayannopoulos, 2018). This mechanism is especially crucial in fighting against pathogens that are too large to be phagocytosed, such as *C. albicans* hyphae (Branzk et al., 2014, Pappas et al., 2018). To assess how each GRACE^{TM} mutant affected the release of NETs, the NETosis assay was performed. The data revealed that all *C. albicans* mutants could induce NETosis, which is consistent with previous observations (Branzk et al., 2014, Urban and Nett, 2019, Urban et al., 2006). However, out of all the GRACE^{TM} mutants, *noc3* appeared to induce significantly fewer NETs than its WT. This result is similar to the *efg1Δ/Δ cph1Δ/Δ* mutant, which induced significantly fewer NETs than its parental control. The fluorescence images also showed that the *noc3* mutant had a morphology closer to the *efg1Δ/Δ cph1Δ/Δ* mutant than the *tup1Δ/Δ* mutant. This evidence, together with its MI score (Table 5.1) and growth rate (Table 4.2), further indicated that the *noc3* mutant has a growth defect and cannot form complete or true hyphae, which emphasises the importance of the *NOC3* gene in morphogenesis. Furthermore, the finding is consistent with a previous study where the authors showed that larger pathogens (such as hyphal units of *C. albicans* and *Aspergillus fumigatus*) induced more NETosis than smaller pathogens (such as yeast units of *C. albicans* and small conidia) (Branzk et al., 2014). My evidence and previous findings suggest that NETosis may be selectively triggered depending on the size of the pathogen. Therefore, the morphology defect of the *noc3* and *efg1Δ/Δ cph1Δ/Δ* mutants could explain why they stimulated fewer NETs. Furthermore, it has been shown that phagocytosis can inhibit

NETosis by downregulating neutrophil elastase (NE) translocation to the nucleus (Branzk et al., 2014). Perhaps, *noc3* and *efg1Δ/Δ cph1Δ/Δ* mutants were cleared by phagocytosis due to their smaller size, and thus fewer NETs were released. In contrast to previous findings, the assay revealed that NETosis triggered by the *tup1Δ/Δ* mutant was significantly lower than its WT control, although this may be because fewer cells of these mutants were added due to difficulties in the calculation of cell numbers. Also, the *tup1Δ/Δ* mutant seemed to have a similar ability to induce NETosis to the *noc3* and *efg1Δ/Δ cph1Δ/Δ* mutants. This disagreement is likely because I might not have added enough *tup1Δ/Δ* cells as previously explained.

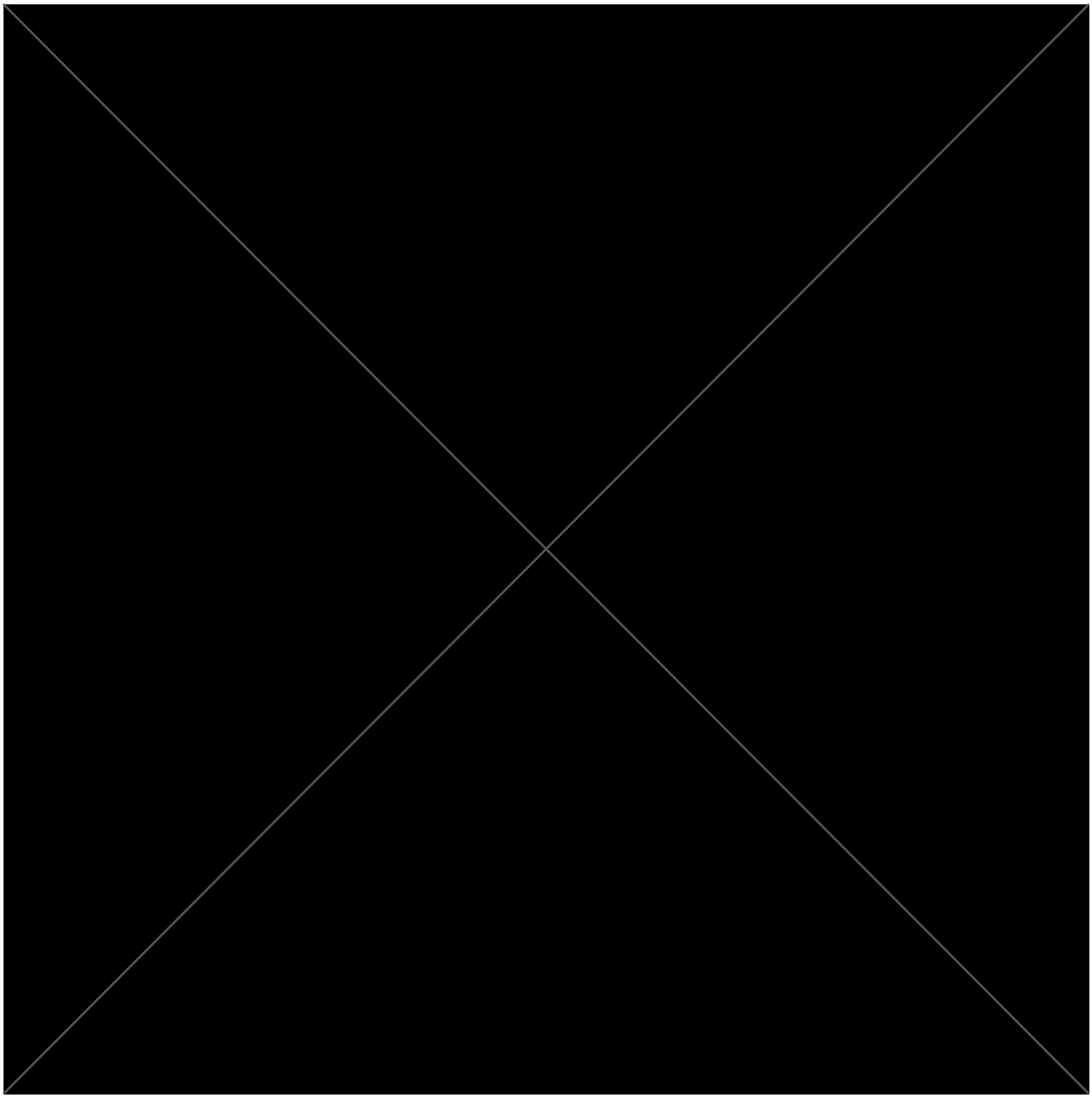
The NETosis assay also revealed an interesting trend for two GRACE^{TM} mutants (*smt3* and *tfb3*), where they consistently induced higher NET release than their WT control (Supplementary figure 5). In addition, it appeared that these mutants could form hyphae but less extensive compared to their WT, as shown by the fluorescence images and previous MI scores. This suggested that *C. albicans* size may not be the only determining factor of NETosis. Perhaps, NETosis induction may depend on how much "NET-specific" PAMPs are presented on the cell wall rather than the actual size of the fungus. For example, the recognition of fungus by dectin-2 has been shown to be the main NETosis driver (Miura et al., 2019, Papayannopoulos, 2018, Wu et al., 2019). Therefore, a strong NETosis inducer mutant (such as *smt3* and *tfb3* mutants) may have a greater abundance of dectin-2 agonists, such as α-mannan (Gazendam et al., 2016, Gow et al., 2012). Furthermore, *Candida* biofilm studies showed that biofilm formation and its extracellular matrix could alter neutrophil recognition and impair NET formation (Johnson et al., 2016, Johnson et al., 2017). Mutants lacking *SMT3* and *TFB3* genes may be defective at producing extracellular matrix and forming a biofilm. This could explain the NETosis trend of these mutants and their susceptibility to neutrophils.

There are some limitations to the NETosis assay that should be taken into consideration when making conclusions. For example, the Sytox™ Orange dye is not specific to NETs as it stained all extracellular DNA. This means that neutrophils that die by *C. albicans*-induced apoptosis will also be detected. Therefore, it is essential to validate this experiment with a more sensitive method, such as fluorescence staining of NET markers: NE translocation and citrullinated histone H3 (Amulic and Sollberger, 2019, Branzk et al., 2014). As *C. albicans* also had a similar Syto™ Green to live neutrophils (those with intact cell membrane), it was impossible to calculate a percentage of NETs, which is a more accurate quantification measure than NPI. To account for the variation of neutrophil responses due to genetic and environmental factors and to avoid bias, it is essential to repeat the experiment with neutrophils from multiple donors.

5.4 CONCLUSION

In this chapter, I have discovered novel characteristics of the five GRACE™ mutants (previously identified in Chapter 4). My project revealed that a mutant lacking the *BRR2* gene had an impaired ability to switch between yeast and hyphae and may be susceptible to H_2O_2 oxidative stress. It also revealed that the fungal cell wall integrity was compromised in the *brr2* mutant. Conversely, the cell wall integrity seemed to be enhanced in a mutant lacking the *TAF7* gene. My study further showed that a mutant lacking the *NOC3* gene appeared to have growth defects and could not undergo yeast-hyphae morphogenesis. The *noc3* mutant also seemed to be a weak inducer of ROS and NET production. Conversely, a mutant lacking the *TFB3* gene appeared to be a strong NET and ROS inducer. Like the *brr2* mutant, my study suggested that the *tfb3* mutant might have an impaired ability to transition between yeast and hyphae and may be susceptible to H_2O_2 oxidative stress. Similarly, a mutant lacking the *SMT3* gene may also have an impaired ability to shift between the two morphologies. In addition, the cell wall integrity of the *smt3* mutant appeared to be compromised and that the mutant seemed to be a weak ROS and a strong NET inducer.

Finally, it is apparent that these five identified genes are crucial for *C. albicans* functions, such as undergoing yeast-hyphae morphogenesis and overcoming neutrophil antifungal responses (Figure 5.9). Therefore, more studies are required to investigate these genetic pathways, which hopefully will lead to identifying novel therapeutic targets for *C. albicans* infections.



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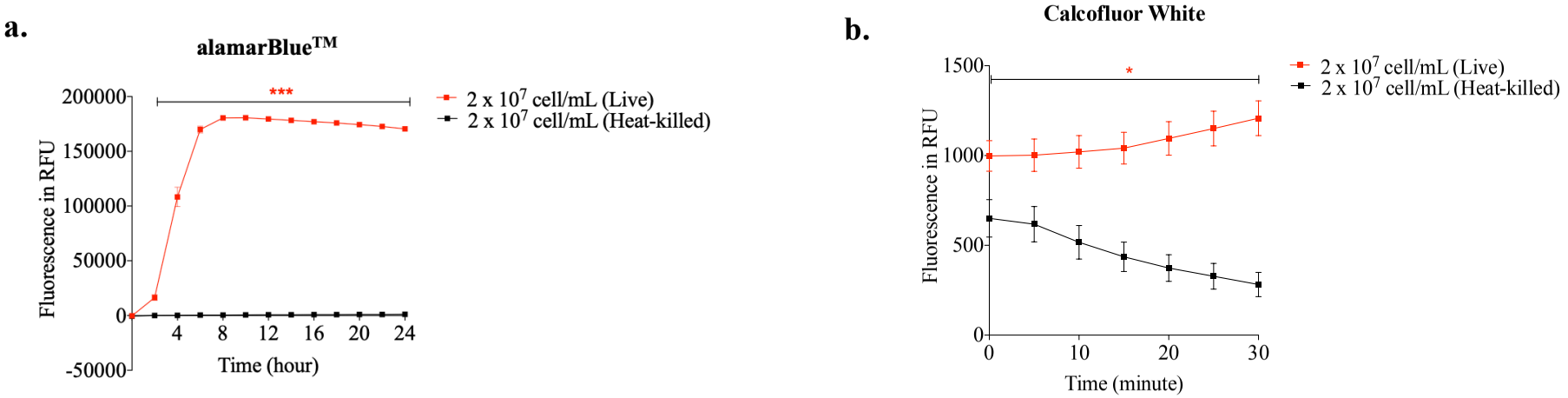
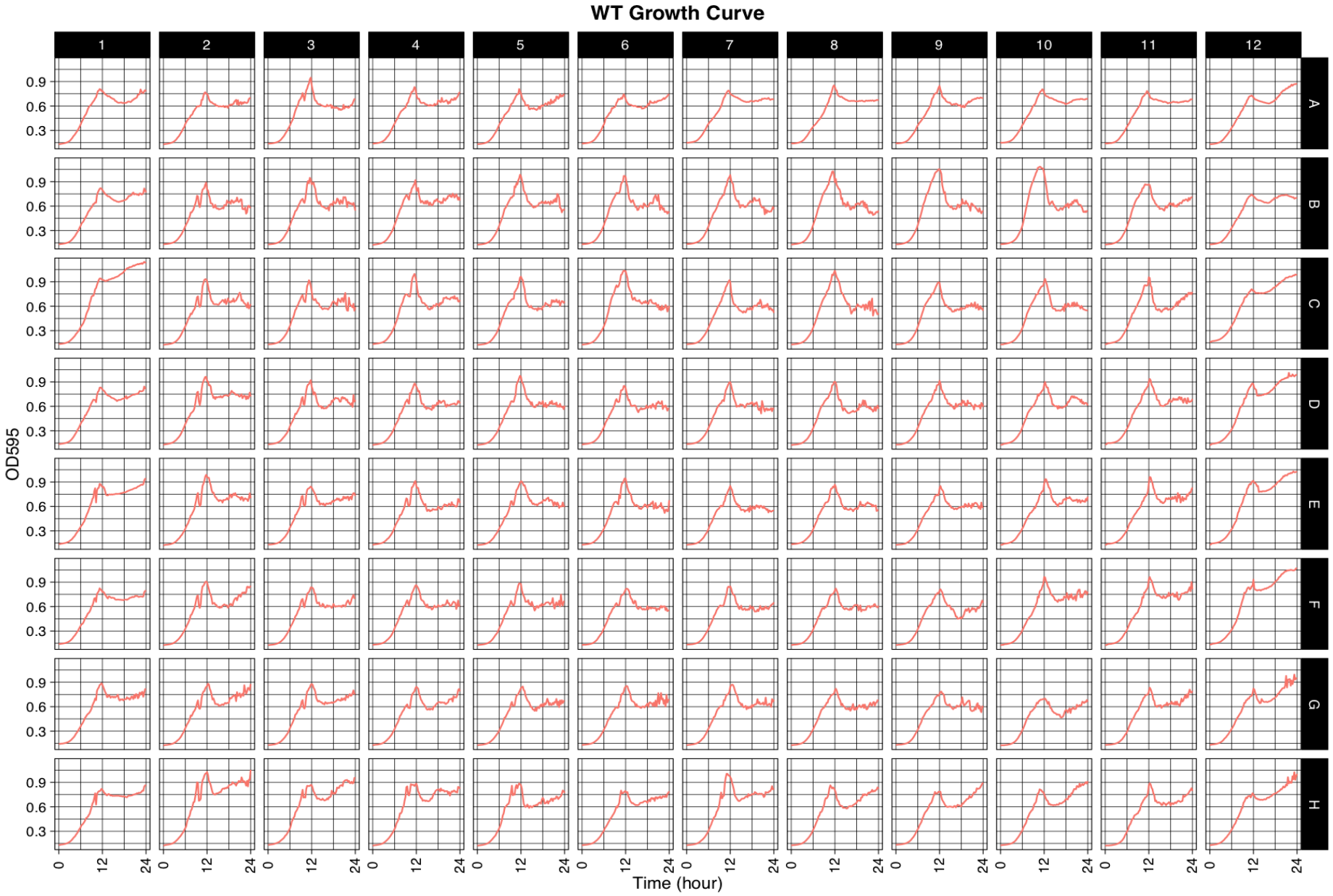
APPENDIX

SUPPLEMENTARY FIGURES

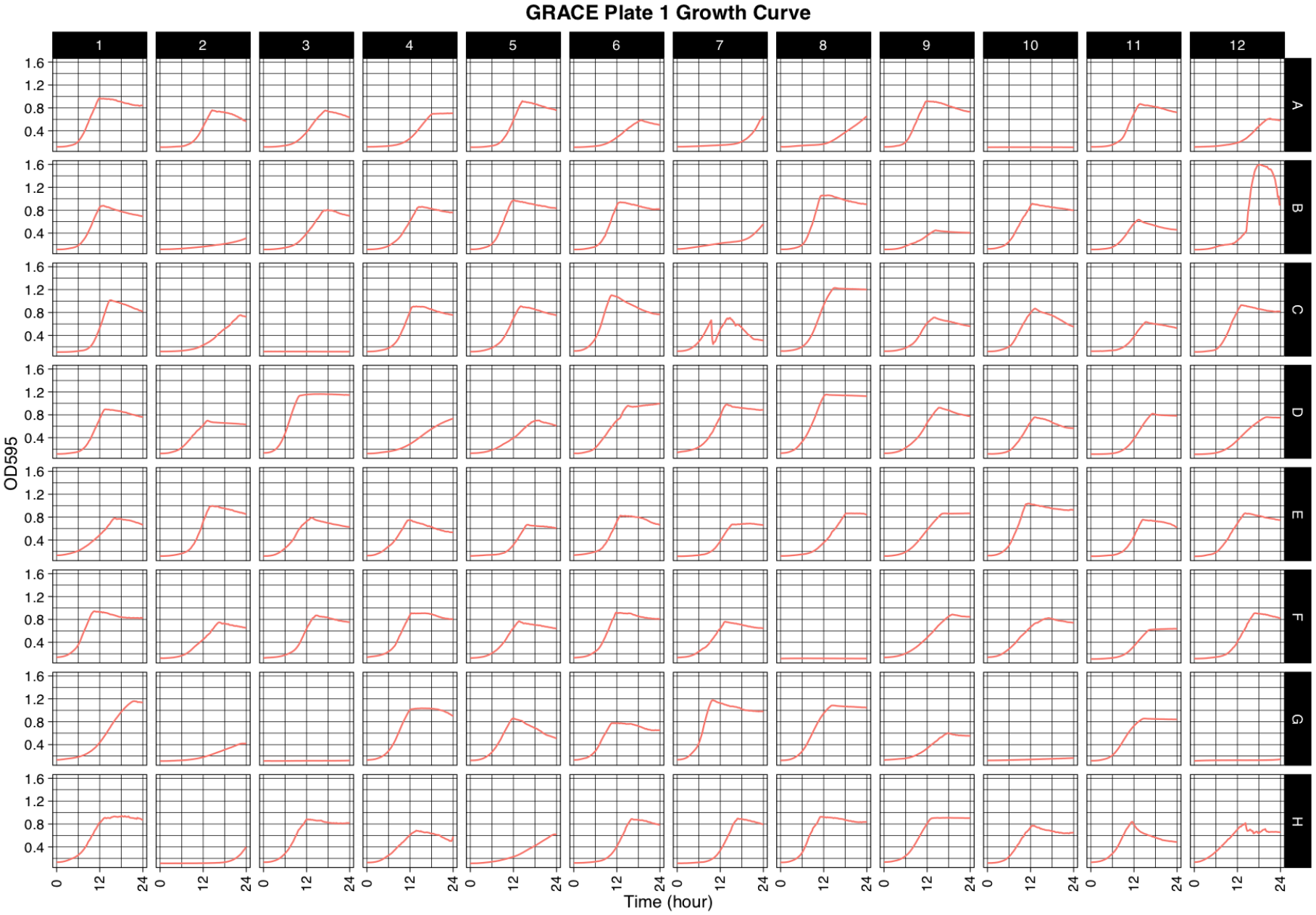


Supplementary figure 1: alamarBlue™ vs. Calcofluor White incubation periods of WT *C. albicans* at 2 x 10^7 cell/mL. (**a.**) alamarBlue™ fluorescence of live and heat-killed cells over 24 hours. (**b.**) CFW fluorescence of live and heat-killed cells over 30 minutes. Fluorescence (mean in RFU) ± SEM. Statistical significance was calculated by 2-tailed multiple paired t-test comparison; * $p<0.05$, *** $p<0.001$; $n=3$.

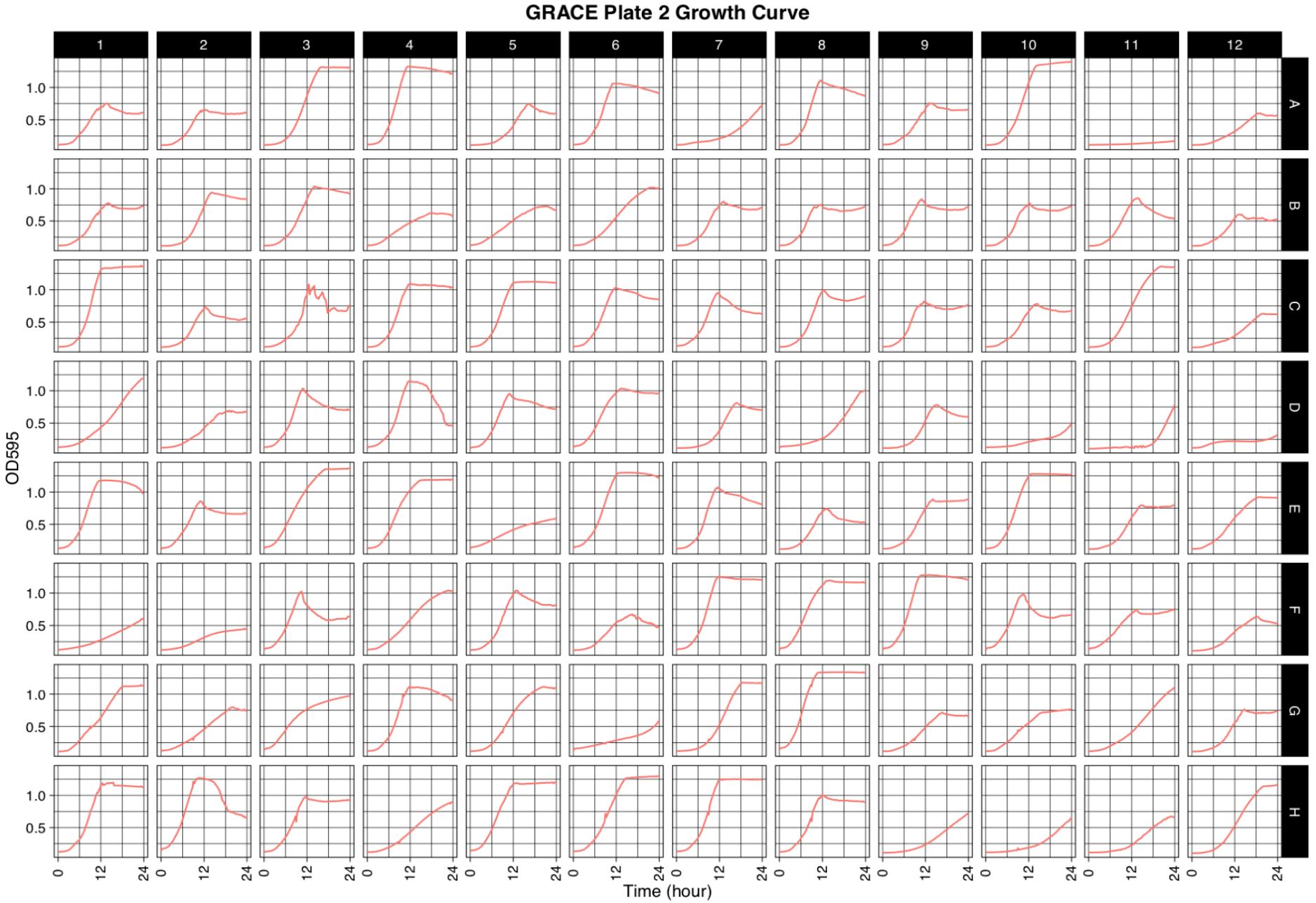
a.


WT Growth Curve
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
OD595
0.9
0.6
0.3
0
12
24
Time (hour)

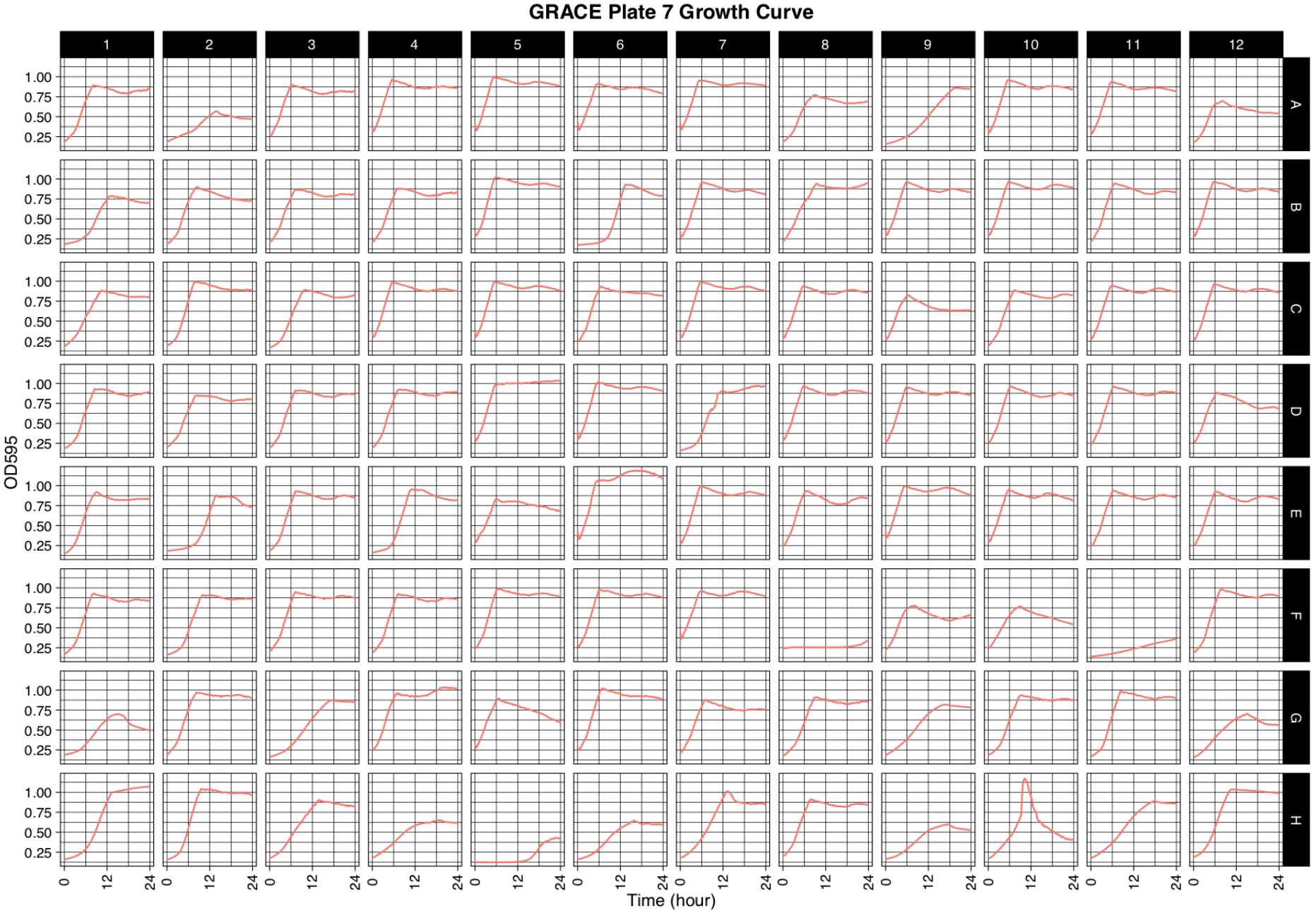
b.


GRACE Plate 1 Growth Curve
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
OD595
1.6
1.2
0.8
0.4
0
12
24
Time (hour)

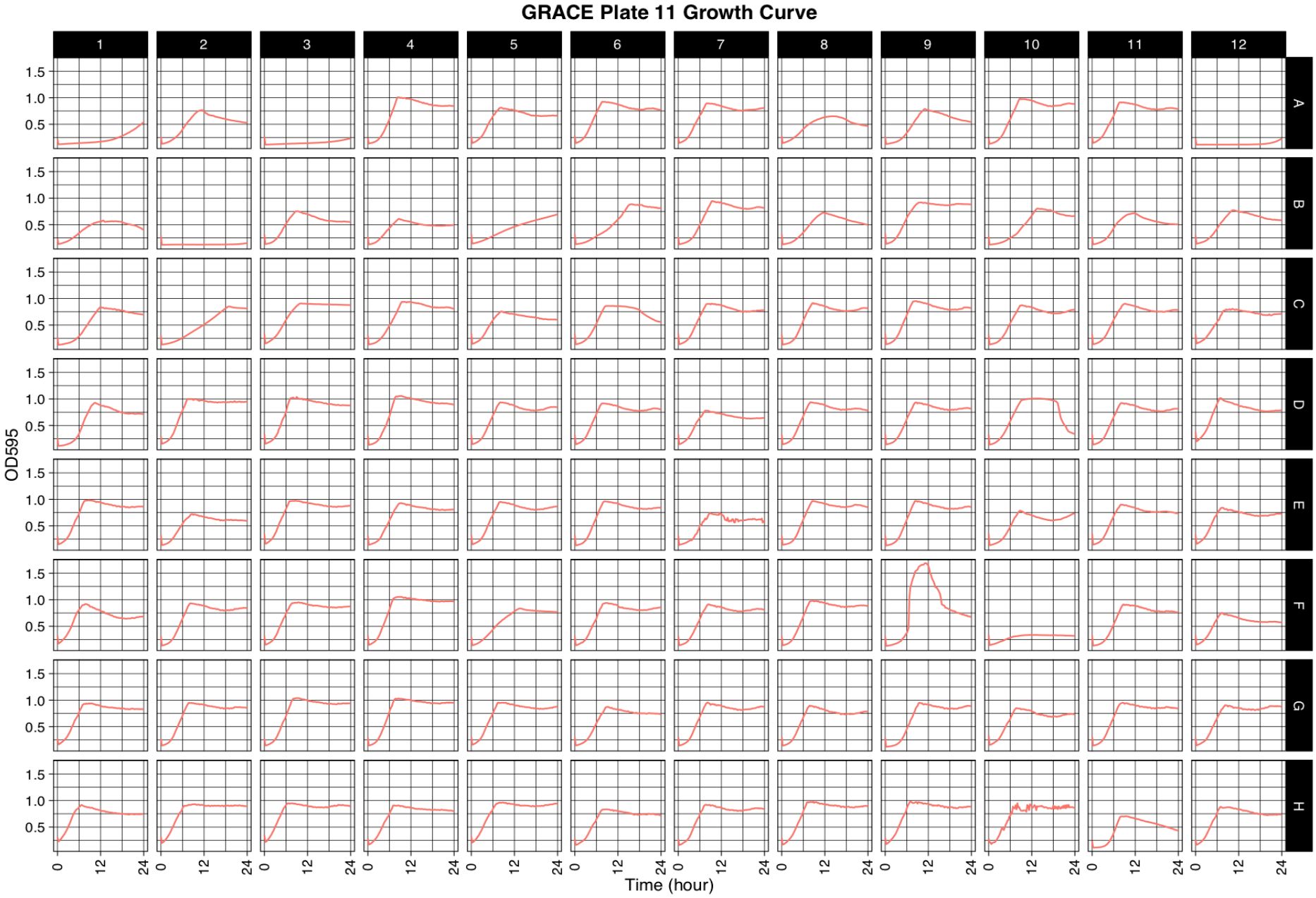
c.


GRACE Plate 2 Growth Curve
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
OD595
1.0
0.5
0
12
24
Time (hour)

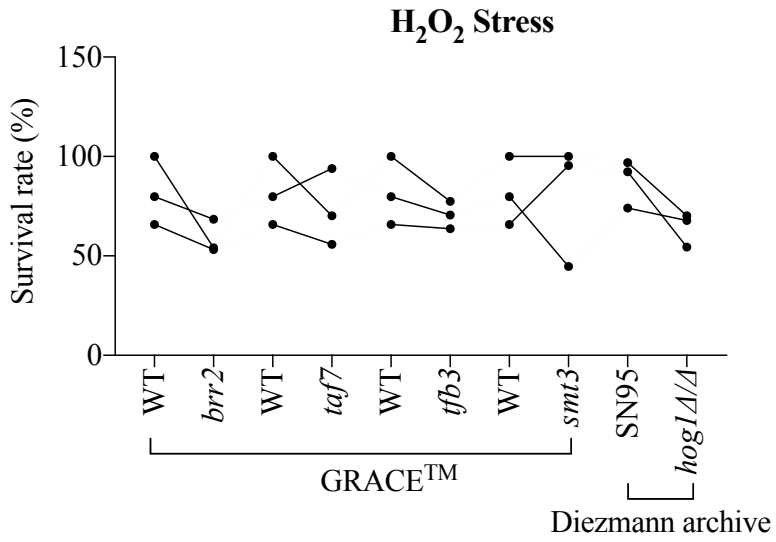
d.


GRACE Plate 7 Growth Curve
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
OD595
1.00
0.75
0.50
0.25
0
12
24
Time (hour)

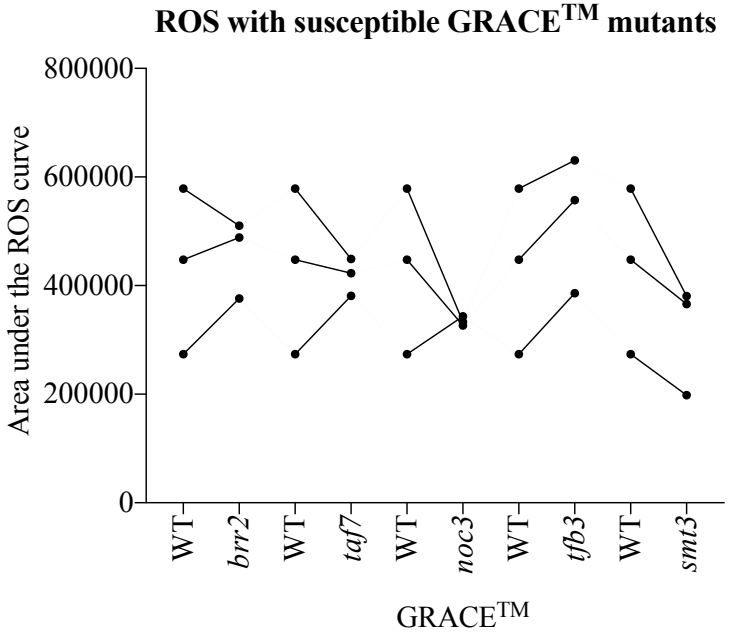
e.


GRACE Plate 11 Growth Curve
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
OD595
1.5
1.0
0.5
0
12
24
Time (hour)

Supplementary figure 2: The GRACE^{TM} library growth curves. (**a.**) WT control. (**b.**) Plate 1 mutants. (**c.**) Plate 2 mutants. (**d.**) Plate 7 mutants. (**e.**) Plate 11 mutants. The GRACE^{TM} library overnight cultures were transferred into clear-96 well plates (filled with 100 µL of YPD) using a 96 long-pin RePad (Singer). The plates were then incubated at 30 ^{o}C and shaking for 24 hours. The OD_{595} of the plate was measured every 15 minutes. The growth curves were plotted using R programming language (codes were developed by Julia Crunden). *n=1*.

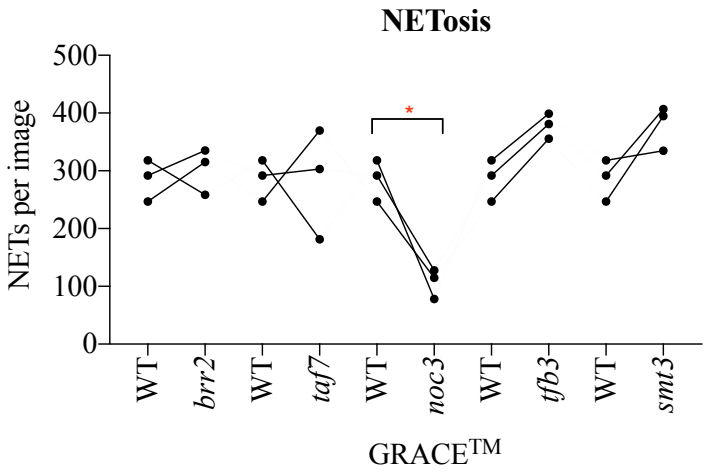


Supplementary figure 3: The GRACE™ mutants may be susceptible to H_2O_2. *C. albicans* - H_2O_2 stress assay was performed, and the survival rate (%) was calculated based on CFU counts (see section 2.2.7). *n=3*.



Supplementary figure 4: Neutrophil ROS production in response to *C. albicans* infection.

The graph shows the area under the ROS curve of the GRACE™ mutants relative to WT. Neutrophils were challenged with *C. albicans* at an MOI of 5 (see section 2.2.9). *n=3* donors.



Supplementary figure 5: **NET production in response to *C. albicans* infection.** The graph shows the number of NETs per image of the GRACE™ mutants relative to WT. Statistical significance was calculated by one-way ANOVA; **p<0.05*, *n=3* donors. Neutrophils were challenged with *C. albicans* at an MOI of 5 (see section 2.2.10).